The Dissertation Committee for Paige Jennette Baugher certifies that this is the approved version of the following dissertation:

Characterization of Metastasis Regulators in Human Breast Cancer:

Implications for Tumor Suppressor PTEN and The Rho Family of Small GTPases

Committee:

_____

Surangani Dharmawardane, Supervisor

_____

Martin Poenie

_____

Kimberly Kline

_____

Bob Sanders

_____

Susan Fisher

Characterization of Metastasis Regulators in Human Breast Cancer: Implications for Tumor Suppressor PTEN and The Rho Family of Small GTPases

by

Paige Jennette Baugher, BMus.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May 2005

For My Dad

Acknowledgements

First, I would like to thank my advisor Su Dharmawardhane for her encouragement, enthusiasm, and **endless** patience during these past six years. I will always admire and love her kindness and generosity toward people, her acceptance and compassion during difficult times, and her love of science.

Also, I would like to thank the love of my life, Jason Hutchings. Nothing but his love for me can rival the kindness and patience he has shown me in the time we have been together. Without him, my goals would have been unattainable and my life unimaginable.

Most of all, I would like to thank my parents. Without their steadfast support and unconditional belief in me and love for me, I would be nothing. From them I have learned never, ever to give up on my goals and dreams, and to face challenges with a balance of determination, dignity, and wit. I have always aspired one day to posses their grace and wisdom, and their ability to know the difference. Thanks, guys.

Characterization of Metastasis Regulators in Human Breast Cancer:

Implications for Tumor Suppressor PTEN and The Rho Family of Small GTPases

Publication No. _____

Paige Jennette Baugher, PhD.

The University of Texas at Austin, 2005

Supervisor: Surangani Dharmawardhane

Cancer metastasis is a multi-faceted process requiring the disregualtion of numerous signaling pathways, including those associated with cell adhesion and motility. Recent data indicates strongly that growth at a primary tumor site and growth at a metastatic site differ by the expression and/or context-dependent function of the metastasis regulator, and that a wide variety of signaling pathways are affected. PTEN (phosphatase and tensin homologue deleted on chromosome ten) then becomes an attractive candidate for a metastasis suppressor, based on its ability to negatively regulate numerous pathways involved in cell survival, cell proliferation, and cell motility. Conversely, the Rho family of small GTPases have become attractive candidates as contributors to metastasis. Rho GTPases regulate numerous signaling pathways involved in cell survival, cell proliferation and cell motility, but they function to enhance these processes instead of inhibiting them.

Data presented here demonstrates the ability of PTEN to negatively regulate motility in human metastatic breast cancer cells without causing the cells to undergo apoptosis. PTEN is localized in stimulated cells away from the leading edge, which displaces it from sites of active motility signaling and prevents it from inhibiting these processes. Furthermore, ectopic PTEN expression is shown to downregulate phosphoinositol (3,4,5) triphosphate (PIP3), expression. Therefore, PTEN could be acting as a metastasis suppressor in human breast cancer.

Data presented here also demonstrate the ability of the Rac subfamily of Rho GTPases to enhance metastatic properties and contribute to metastasis. Increased Rac activity was shown to correlate with increased metastatic potential in a panel of metastatic human breast cancer cell variants. When activated Rac1 or Rac3 was expressed stably in the least metastatic variant, either isoform was found to enhance adhesion, migration, and invasion *in vitro*, as well as contribute to pulmonary metastasis in the nude mouse model of experimental metastasis. Conversely, when dominant negative Rac1 or Rac3 was expressed in the most metastatic variant, either isoform was found to decrease adhesion, migration, and invasion *in vitro*, as well as block pulmonary metastasis *in vivo*. Therefore, Rac1 and/or Rac3 are found to act as metastasis regulators by negatively regulating metastatic human breast cancer progression.

TABLE OF CONTENTS

page

1. Introduction

1.1 Cancer

Cancer is a general term that describes a group of diseases characterized by uncontrolled cellular growth, or neoplastic transformation. Neoplastic transformation is a multi-step process that requires multiple genetic changes. If unchecked, neoplastic transformation can result in the formation of a tumor, or an abnormal mass of cells. Tumors have the potential not only to invade and destroy surrounding tissue, but also spread through the blood stream and establish secondary tumors at distant sites. Because cancer can occur in most any tissue in the body, there are more than a hundred distinct types of this disease (Cooper and Hausman, 2004). These cancer types can vary substantially in behavior, protein expression, and eventual response to treatment. However, the cells that comprise tumors share three basic biological properties: uncontrolled (density-independent) proliferation, impaired cellular differentiation, and invasiveness (Karp, 1999). Recent research has revealed that all cancers share common molecular mechanisms governing these biological properties, and those molecular mechanisms have become the first line of attack in order to find treatments or cures for the disease.

Oncogenes and Tumor Suppressors

Because it can be traced to mutations within specific genes that lead to abnormal gene expression, cancer is considered a genetic disease. Basically two types of genetic mutations are associated with oncogenic transformation: gain of function or loss of function. Oncogenes are genes that are mutated in such a way as to cause gene product overexpression or hyperactivation (gain of function mutation). These genes typically encode proteins important for cell cycle progression or cellular proliferation, and consequently cause cellular transformation and tumor formation when hyperactivated. Conversely, tumor suppressor genes encode proteins that negatively regulate cellular growth. These genes are mutated in such a way as to inactivate, or incapacitate, the encoded protein (loss of function mutation). As these proteins negatively regulate cell proliferation, loss of protein function can result in uncontrolled proliferation, or cellular transformation. Generally, the more advanced a cancer is, the more genes that have been altered within the cells. This observation renders cancer extremely difficult to treat for the reason that each tumor has its own profile of oncogene expression and tumor suppressor inactivation. Essentially, each tumor is genetically unique and therefore will respond uniquely to different treatments.

Causes of Cancer

A tumor that is invasive is referred to as a carcinoma, or cancer. The causes of cancer can be divided into two groups: exogenous (environmental factors) and endogenous (genetic predisposition). Exogenous carcinogens cause sporadic cancers that

are caused by a cumulative series of somatic mutations acquired over the lifetime of the patient. Hereditary cancers, or cancer predisposition syndromes, represent a type of tumor formed from a genetic mutation that is inherited. However, inherited genetic alteration is rarely enough to cause tumors. Disease is often presented only after exposure to environmental insults.

Carcinogenesis is thought to develop in two stages: initiation and promotion. The first stage of carcinogenesis, or initiation, involves the mutation of nuclear DNA by a mutagen. However, this mutation is not enough to cause uncontrolled cellular growth. The cell containing the mutation must then be unable to police the damaged DNA, and so replicate with no DNA repair. The mutated cell must also lose the ability to control proliferation. This latter stage is referred to as "promotion", and requires repeated exposure of the cell to a promotion agent. Tumor promoters do not cause tumorigenesis alone, but enhance tumor formation subsequent to mutagenesis.

Breast Cancer

Breast cancer is the leading type of cancer occurring in women in the US, and it affects almost one million women worldwide at any give time (Bowcock, 1999). It is estimated that one in eight women will develop breast cancer, and of these women, 30% will die from metastatic progression (Bowcock, 1999). The American Cancer Society predicts that in the year 2005, 40,000 women will die from this disease (ACS, 2005).

Breast cancer is a malignant tumor that has developed from the cells of the breast.

There are essentially three distinct structures that make up the female breast: the lobules, or milk-producing glands; the ducts, or the passages that connect the lobules to the nipple and conduct milk during lactation; and the stroma, or the fatty and connective tissue that surrounds the ducts and lobules, blood vessels, and the lymphatics. Nearly all breast cancers arise in either ductal tissue or lobular tissue, and are referred to as adenocarcinomas. The most common diagnosis for aggressive breast cancer is Invasive Ductal Carcinoma (IDC). This cancer has arisen from epithelial cells lining the milk ducts, but it has invaded into the wall of the duct itself. This type of cancer will account for about 80% of invasive breast cancer diagnoses (ACS, 2005).

Studies have shown this high risk in part due to the structure and development of the mammary gland itself (Russo et al., 2001). The breast undergoes dramatic changes in size, shape, and function, depending on growth, reproduction, and menopause (Russo et al., 2001). Essentially, the breast was designed to fluctuate dramatically in size, rendering it more susceptible to uncontrolled proliferation than other organs that do not experience such turnover. Interestingly, cancer risk appears to be related to the duration of the periods of homeostasis (Anderson, 2004). Encouragingly, mortality from breast cancer is decreasing due to early detection (ACS, 2005). However, metastatic disease almost always ends in death. Therefore, focus must be realigned with breast cancer metastasis, and the mechanisms by which this process occurs.

Incidence and Mortality Statistics

Cancer not only presents an interesting molecular mechanism challenge, but it also presents a terrible and debilitating disease. In 2002, the American Cancer Society ranked cancer as the second leading cause of death, accounting for nearly 25% of all deaths in the United States (ACS, 2005). By 2001, it was estimated that 1 in 2 men would be diagnosed with cancer in their lifetime, while those statistics are 1 in 3 for women (ACS, 2005). Moreover, cancer has seen the least advancement in prevention and cure among the top four leading causes of death in the US in the last 50 years (ACS, 2005). Clearly, cancer presents an acute problem with relatively slow success in treatment advancements.

1.2 Metastasis

Tumors, or masses of cells resulting from uncontrolled cellular growth, fall into two general categories: benign and malignant. Benign tumors place the patient a low risk, for they have not yet invaded the surrounding tissue and have a low probability of spreading throughout the body. Malignant tumors, or carcinomas, have invaded the surrounding tissue and have a high probability of spread. Whereas benign tumors can usually be removed surgically due to well-circumscribed borders and confinement to the original tissue, malignant tumors present more of a problem. If the tumor has spread to

other parts of the body, surgery is usually not an option. Treatment becomes more difficult, more dangerous, and mortality dramatically increases.

Metastatic Progression

Metastatic progression is the process by which cancer spreads throughout the body. Cancer cells, migrating as individuals or as aggregates, actively migrate away from the primary tumor, invade through the surrounding extracellular matrix, and enter the blood stream. These cells then are carried via the circulatory system to distant sites, where the cells exit the blood stream and form secondary tumors at these sites. By definition, metastatic cells must have acquired more mutations than primary tumor cells. Metastatic cells must not only have acquired the ability to proliferate uncontrollably, but must have acquired the ability to actively migrate and invade into surrounding tissue. In the case of most breast cancers, the tumor arises from well-differentiated, polarized epithelial cells. The cells must undergo the epithelial to mesenchymal transition to acquire the ability to attain fibroblast-like motility and invasion needed for metastatic progression. Consequently, metastatic cells must either acquire gain of function mutations in genes important for cell motility and invasion, or loss of function mutations in genes that negatively regulate these processes.

Surprisingly, only a few steps of metastasis are rate-limiting (Chambers et al., 2002). Studies have shown high numbers of cancer cells in the circulatory system of cancer patients as compared with the number of metastases in distant organs (Chambers et al., 2000). Furthermore, cell survival in the circulation, arrest in distant organs, and

initial extravasation were found to be relatively efficient (Steeg et al., 2003). However, the metastatic colonization of distant organs was shown to occur quite inefficiently, and appears to be the rate-limiting step of metastasis (Chambers et al., 2000).

Metastasis Suppressors/Inhibitors

Recently, a class of genes labeled "metastasis suppressors" is beginning to gain attention. They have been identified by reduced expression in metastatic tumors as compared to their primary, non-metastatic, counterparts (Steeg et al., 2003). Metastasis suppressors appear to act at different steps of the metastatic process, and are not just limited to suppress the rate-limiting metastatic colonization (Steeg et al., 2003). One of the first identified metastasis suppressors, NM23, has been shown to reduce Extracellular-Signal-Regulated-Kinase-Mitogen-Activated-Protein-Kinase (ERK) activation levels, as compared to controls (Steeg et al., 2003). ERK is a member of the Mitogen Activated Protein (MAP) kinase family of serine-threonine kinases that initiate phosphorylation signaling cascades which eventually result in the initiation of cell proliferation (Rubinfeld and Seger, 2004). Conversely, metastasis suppressor RhoGDI2 (Rho Guanine Dissociation Inhibitor 2) acts on adhesion and motility pathways (Gildea et al., 2002). Therefore, it is difficult to predict the efficacy of future metastasis suppressors without extensive investigation.

1.3 Cell Motility

Metastatic progression is thought to be facilitated by cell motility. Cell motility is a broad term that encompasses the migration of cells across a substrate, the ability of cells to make and break adhesions with the substrate across which they are moving, and invasion of cells within a three-dimensional matrix. Most early work with mammalian cell motility was accomplished by observing cells crawl across two-dimensional substrates (usually coverslips), and this is how the process is best understood. At its most basic, cell movement can be divided into three distinct steps: extension of the leading edge, attachment of the leading edge to the substratum, and retraction of the rear of the cell (Alberts et al., 2002). However, motility is a highly complex and intricately regulated process. Different parts of the cell must change at the same time, and there is no single gene, set of genes, or apparatus responsible for mammalian cell migration (Alberts et al., 2002). The most important protein regulating cell motility is actin, a highly abundant cellular protein that polymerizes to form cytoskeletal filaments.

Actin

Actin exists in cells as both globular (g-actin) and filamentous (f-actin). G-actin polymerizes into filaments either spontaneously *in vitro* if monomer concentration and ionic strength is optimal, or assisted by a myriad of actin-binding proteins *in vivo*. Whether *in vivo* or *in vitro*, actin polymerization results in a polarized filament. Even though both ends are capable of adding or subtracting monomers, the plus end (or barbed

end), is more likely to add actin monomers than the minus end. Actin monomers hyrdolyze ATP when incorporated into filaments, and actin monomers bound to ATP are more likely to polymerize than those bound to GDP, creating this polarization.

There exist in the cell a plethora of actin-binding proteins that regulate assembly and disassembly of actin filaments; in fact, turnover of actin filaments within the cell is 100 times faster than it is *in vitro* (Cooper and Hausman, 2004). A key complex of proteins, the Actin-Related Protein 2/3 (Arp2/3) complex, regulates the initiation of the polymerization of new actin filaments (Higgs and Pollard, 2001). This complex functions by binding to existing actin filaments and acting as a nucleation site for a new filament (Pollard and Beltzner, 2002). The discovery of this protein complex unlocked the mystery of not only actin polymerization *in vivo*, but also actin branching. In addition to actin branching, actin filaments are also cross-linked to form a meshwork necessary for the stability of the cell cortex and actin-based cell protrusions. Three actin-based structures are linked to cell motility: lamellipodia, filopodia, and stress fibers.

Lamellae

Lamellipodia, or membrane ruffles, have been referred to as the "organelle" of motility (Abercrombie et al., 1970). They are found at the leading edge of migrating cells during directed motility, and consist of an intricate network of crosslinked actin (Matsudaira, 1994). Membrane protrusion is based on active actin polymerization; in fact, these structures account for the most incorporation of unpolymerized actin in the cell (Glacy, 1983). Also found in lamellipodia are sites of contact between the cell and its

substratum (Kaverina et al., 2002). These contact sites are thought to be important for force generation in the migration process (Beningo et al., 2001). Clearly, lamellipodia are important for cell migration, and recent evidence has linked them to malignant invasion as well (Condeelis et al., 2001).

Filopodia

Filopodia, or membrane spikes, are also tightly linked to cell motility. Also found at the leading edge of migrating cells, filopodia consist of actin filaments aligned in parallel bundles (Wood and Martin, 2002). These actin structures are generally thought to function in sensing environmental cues to guide cell migration and lamellipodia formation (Wood and Martin, 2002).

Stress Fibers

The final actin structure linked to cell motility is the stress fiber. Stress fibers are parallel bundles of actin filaments that provide structure and rigidity to the cell. An overabundance of stress fibers is generally associated with a stationary cellular morphology, while the moderate presence of these structures is necessary for motility (Vial et al., 2003). Stress fibers provide a structure necessary for actin-myosin contraction and the retraction of the cell body during migration (Katoh et al., 2001). However, numerous stress fibers restrain the cell and negatively regulate any forward movement.

Focal Adhesions (FAs) and Focal Adhesion Complexes (FACs)

Stress fibers and actin filaments end in points of contact between the cell and the substratum. These types of contacts are not only sites that provide structural integrity, but they also represent scaffolding sites for cell signaling proteins to congregate and function (Wozniak et al., 2004). The nomenclature is difficult to pinpoint, for there are several different types of these structures, each with its own specific protein complement found throughout the cell (Geiger et al., 2001). Essentially, these structures link actin filaments with proteins of the extracellular matrix through the transmembrane proteins integrins. Integrins directly contact the extracellular matrix proteins, but require adaptor proteins to bind actin (Wozniak et al., 2004). These adaptor proteins function to provide structural support for the cell-matrix junction.

There can be upwards of 100 proteins in cell-matrix adhesion sites, and many of these proteins function as signaling molecules (Geiger et al., 2001). Signaling molecules such as Focal Adhesion Kinase (FAK) and the Rous sarcoma virus gene, Src, are recruited to active integrins, and begin a myriad of signaling cascades (Petit and Thiery, 2000). FAK initiates cell survival and cell proliferation pathways, as well as those associated with cell motility (Mitra et al., 2005). Src is important for survival signaling, as well as cell cycle progression (Parsons and Parsons, 2004). In fact, if an untransformed cell is in suspension or devoid of matrix contacts, it will undergo apoptosis, or anoikis (Zhan et al., 2004).

Invasion

Cell invasion refers to the process by which a cell moves through a three-dimensional matrix. Not only is it necessary for the cell to form actin protrusions, make cell-matrix contacts, and retract, but the cell must also clear a path for itself to migrate through the maze of interwoven extracellular matrix proteins. To do this, the cell secretes matrix metalloproteinases (MMPs) that digest the proteins of the extracellular matrix. As a group, MMPs have the ability to digest essentially all protein components of the extracellular matrix (Kleiner and Stetler-Stevenson, 1999). Degradation of the basement membrane, or the extracellular matrix proteins underlying sheets of epithelia, is essential for tumor cell intravasation (Overall and Lopez-Otin, 2002). Recently, it has become evident that the MMP family plays a direct role in tumor progression by regulating the tumor microenvironment (Egeblad and Werb, 2002). Moreover, MMP expression is increased in most human cancers compared with normal tissue (Egeblad and Werb, 2002). MMP inhibition is currently an active area of study for cancer treatment (Folgueras et al., 2004).

1.4 Rho GTPases

The Rho GTPases are a family of proteins essential for cell motility. This family consists of 20 proteins, and can be subdivided into five subfamilies that exhibit similar properties: the RhoA-related subfamily, the Rac1-related subfamily, the Cdc42-related subfamily, the Rnd subfamily, the RhoBTB subfamily (Burridge and Wennerberg, 2004).

Additionally, there are three proteins RhoD, Rif, and TTF/RhoH, which do not fall into any of these subfamilies (Wennerberg and Der, 2004). Very little is known about these proteins, and it is questionable whether they are important to cancer progression (Burridge and Wennerberg, 2004). The function and signaling of the RhoBTB proteins is completely unknown, and therefore not currently pertinent to metastatic progression (Burridge and Wennerberg, 2004). Rnd subfamily proteins are closely related to the RhoA subfamily, but seem to antagonize Rho signaling and lead to cell rounding (Nobes et al., 1998). Again, the role of the Rnd subfamily in the positive regulation of metastatic progression is questionable, and therefore not currently relevant.

The most intensely studied Rho GTPases are the RhoA-like, Rac1-like, and Cdc42-like subfamilies. There is much evidence that proteins of these subfamilies play significant roles in tumor progression to the metastatic state (Benitah et al, 2004, Ridley, 2004; Sahai and Marshall, 2002). Members of these families have been shown to be overexpressed in human tumors and cancer-associated mutations in Rho protein regulators have been characterized (Ridley, 2004). Furthermore, Rho family proteins have been shown to be important for the proper maintenance of epithelial cell-cell adhesion (Lozano et al., 2003). Rho proteins have been implicated not only in motility pathways, but in cell cycle progression and cell proliferation as well (Benitah et al., 2004).

RhoA Subfamily

The RhoA-like subfamily consists of RhoA, RhoB, and RhoC. RhoB regulates actin organization and vesicle transport, but has been shown to possess anti-cancer function (Prendergast, 2001). However, RhoA and RhoC are strongly implicated in cancer progression (Wheeler and Ridley, 2004). RhoA has been found to be overexpressed in several highly metastatic cancer cell lines and can promote transformation of cultured mouse fibroblasts (Ridley, 2004). Conversely, RhoC cannot promote fibroblast transformation, but has been shown to increase in expression levels as tumors become increasingly metastatic (Clark et al., 2000; Kleer et al., 2002). Additionally, RhoC has been shown to promote metastasis when overexpressed in melanoma cells (Clark et al., 2000).

It is becoming more evident that even though RhoA and RhoC are very closely related in cDNA sequence, they function differently *in vivo* (Wheeler and Ridley, 2004). RhoA and RhoC have been shown to activate the formin Diaphanous (mDia), which results in actin nucleation that contributes to stress fiber formation (Wallar and Alberts, 2003). Additionally, they have also been shown to activate Rho Kinase (ROCK), a kinase that elevates Myosin Light Chain (MLC) phosphorylation (Sahai and Marshall, 2002). This elevation causes acto-myosin contraction within the cell, which is an indispensable step in cell migration (Burridge and Wennerberg, 2004). Interestingly, RhoC appears to exhibit a higher affinity for ROCK and a stronger ability to activate it than its isoform, RhoA (Sahai and Marshall, 2002). This difference could explain the different roles played by each isoform in cell motility and cancer progression.

Rac1-like and Cdc42-like Subfamilies

The Rac1-like subfamily includes three members, Rac1, Rac2, and Rac3. Rac2 expression is restricted to hematopoetic cells where is required for function of the NADPH oxidase (Dinauer, 2003). Rac1 and Rac3, exhibiting 92% homology, stimulate the formation of lamellipodia and membrane ruffles (Aspenstrom et al., 2004). Rac1 has been strongly implicated in metastatic progression, while a role for Rac3 is beginning to become evident (Sahai and Marshall, 2002). Activating Rac1 causes an increase in lamellipod expression and an increase in invasion in transformed, but non-invasive, cells, implicating it in tumor progression (Bourguignon et al., 2000). Additionally, blocking Rac1 function curtailed metastasis in an *in vivo* model (Bouzahzah et al., 2001). A similar role in metastasis for Rac3 has not been shown, even though Rac1 and Rac3 share similar downstream effectors (Haeusler et al., 2003).

The Cdc42-like subfamily consists of five members, and they all stimulate the formation of filopodia (Burridge and Wennerberg, 2004). However, Cdc42 is the most extensively studied protein of the family and has been implicated in cancer progression (Schmitz et al., 2000).

Rac and Cdc42 share most of the same downstream effectors, due to extensive homology of the effector region (Cotteret and Chernoff, 2002). The major exception to this observation is the ability of Cdc42 to bind Wiskott-Aldrich Syndrome Protein (WASP) *in vivo*. Subsequent to activation by Cdc42, WASP activates Arp2/3, which causes actin nucleation and the formation of filopodia (Miki and Takenawa, 2003). Ironically, Rac can also activate Arp2/3 *in vivo* to result in actin polymerization, but

through the protein WASP family verprolin homologous (WAVE) (Smith and Li, 2004). However, this interaction is not direct: Rac1 instead directly interacts with either insulin receptor substrate 53 (pIRS53), or the protein complex consisting of WAVE1, p-53 inducible messenger RNA with a relative molecular mass of 125,000 (PIR121), NCK-associated protein with a relative molecular mass of 140,000 (Nap125), and Heat Shock Protein 300 (HSPC300), to activate WAVE (Eden et al., 2002).

Downstream effectors shared by Rac and Cdc42 include p-21 activated kinase (PAK), PI3-kinase (phosphatidyl inositol 3-kinase), and members of the MAP kinase cascades, MEKK1, MEKK4, and Mlks 1,2,3 (Bishop and Hall, 2000). The most intensely studied downstream effector common to both Rac and Cdc42 is PAK. PAK can stimulate cell migration via LIMK, filamin, or its effects on myosin (Bokoch, 2000). Additionally, PAK can activate p38MAP kinase and Jun kinase (JNK), which can lead to cell proliferation (Bishop and Hall, 2000). Recent studies have even implicated PAK in human cancer (Vadlamudi and Kumar, 2003).

Regulation

The Rho GTPases Rho, Rac, and Cdc42 are all active when bound to GTP, and inactive when bound to GDP. When bound to GDP, Rho proteins are sequestered in the cytosol by RhoGDI (guanine nucleotide dissociation inhibitor), which masks the prenyl group and prevents translocation to the plasma membrane. Subsequent to RhoGDI dissociation, Rho proteins translocate to the plasma membrane where they are activated by guanine nucleotide exchange factors (GEFs). GEFs allow the binding of GTP to the

Rho protein by facilitating the dissociation of the GDP. Rho GTPases have intrinsic GTPase ability, but this ability is very weak. In order to hydrolyze the gamma phosphate of the GTP to render the Rho GTPase inactive, a GTPase activating protein (GAP) is required. Clearly, the Rho GTPases are intricately regulated by a myriad of accessory proteins.

Structure, Biochemical Interactions

The Rho GTPases Rho, Rac, and Cdc42 all contain similar activating and structural domains. The GTPase binding domain is located near the N-terminus. The effector domain, or Switch I region, is located between residues 28-44 in RhoA. This region undergoes a conformational change when GDP is exchanged for a GTP, and this conformational change allows the binding of downstream effectors. Proteins containing Cdc42 and Rac interactive binding (CRIB) domains or GTPase binding domains (GBDs) bind to the Rho proteins at the Switch I region, which is located close to the N-terminus of the protein. C-terminal to Switch I is the Insert region. The insert region (residues 124-135 of Rac1) is the most varied among Rho, Rac, and Cdc42 proteins (Freeman et al., 1996). Even though both downstream effectors and GEFs interact with the Switch I domain, the insert region also determines binding affinity of these proteins because accessory proteins interact with other domains as well (Schmidt and Hall, 2002). Finally, Rho proteins contain a CAAX box at their n-terminus where these proteins are prenylated. RhoA and RhoC are farnesylated, Rac1 and Cdc42 are geranylgeranylated, while Rac3 appears to be both farnesylated and geranylgeranylated (Joyce and Cox,

2003). Overall, slight differences within the structure and amino acid sequence of these proteins can account for considerable differences in cellular effects.

Rho Proteins and their Regulators as Potential Cancer Therapeutics

Because Rho GTPases may act as promoters of metastasis, it has long been thought that drugs which specifically alter Rho protein signaling could have significant therapeutic value (Martin, 2003). Because these proteins affect tumorigenesis at various levels, including G1-S transition, cell survival, motility and invasion, it follows that inhibition of these proteins or downstream effectors could be effective anti-cancer therapies. Recently, a group ahs developed a first generation of compounds that target the activity of PAK, a downstream effector of Rac and Cdc42 shown to be necessary for Ras-induced transformation (Nheu et al., 2002). Another area of potential therapy currently being explored is the treatment of human tumors that overexpress Rho GTPases with non-steroidal anti-inflammatory drugs (NSAIDS) (Benitah et al., 2003). The NSAIDs Sulindac and NS-398 have been shown to decrease proliferation in human tumor cells via inhibition of the Rho GTPases (Benitah et al., 2003). Furthermore, a group of anti-hypercholesterolemia drugs known as statins have recently been shown to possess anti-tumor effects via the inhibition of the Rho GTPases (Jakobisiak and Golab, 2003).

The greatest successes with anti-cancer agents that target small GTPases have been then farnesyl transferase inhibitors (FTIs). Tipifarnib (R115777), in particular, has enjoyed reasonable success in phase I California Cancer Consortium Trial (Lara et al., 2005). The mechanism of activation of FTIs is to inhibit farnesylation of GTPases,

thereby interfering with translocation to the membrane and effective activation (Caponigro, 2002). Clearly, there is a wealth of possibilities for the development of new anticancer drugs that target Rho GTPases.

1.5 PTEN

PTEN (phosphatase and tensin homologue deleted on chromosome ten) is a tumor suppressor gene that may also act as a metastasis suppressor. It encodes a dual-specificity phosphatase that has been shown to dephosphorylate protein substrates *in vitro* on serine, threonine, and tyrosine residues (Myers et al., 1997). Additionally, PTEN has been shown to dephosphorylate lipids (Maehama and Dixon, 1998). PTEN has come to represent an extremely important protein not just because of its role as a tumor suppressor, but also because of its role in embryonic development, cell migration, and apoptosis (Yamada and Araki, 2001). PTEN has emerged as a significant modulator of cell signaling, growth, migration, as well as apoptosis.

PTEN as a Tumor Suppressor

By the mid-1990's, genetic evidence strongly suggested a tumor suppressor was located on chromosome 10 of the human genome (Parsons and Simpson, 2004). One alteration that was found to occur at high frequency in a variety of human tumors was the loss of heterozygosity at chromosome 10q23 (Tamura et al., 1999). Therefore, the search began for a novel tumor suppressor gene from that region. In 1997, three independent

research labs cloned a tumor suppressor gene from region 10q23, and was referred to as PTEN, MMAC1 (mutated in multiple advanced cancers) or TEP1 (transforming growth factor beta-regulated and epithelia cell-enriched phosphatase) (Waite and Eng, 2002). Not only is PTEN found to be deleted or mutated in many types of somatic cancers, but germline mutations in the gene have been found in individuals with Cowden Syndrome (Waite and Eng, 2002). Cowden Syndrome is an autosomal dominant disorder that is characterized by multiple hamartomas that affect derivatives of all three germ layers and by a risk of breast, thyroid, and endometrial neoplasias (Eng, 2000). Notably, this finding represented the first phosphatase gene that had been implicated in the etiology of an inherited cancer syndrome (Waite and Eng, 2002). Undoubtedly, PTEN is a fascinating protein with great clinical, as well as historical, significance.

Structure, Biochemical Interactions

The crystal structure of PTEN was solved in 1999, which shed insights into its phosphatase activity and membrane association (Lee et al., 1999). Essentially, the PTEN structure consists of the N-terminal phosphatase domain (179 residues) and the C-terminal C2 domain (166 residues) (Lee et al., 1999). The phosphatase domain contains the active site pocket, which contains a P loop that is similar to those found in other protein tyrosine phosphatases (PTPs) (Lee et al., 1999). Unlike these other phosphatases, however, this pocket contains an extension that widens the pocket and allows the binding of the larger lipid substrates (Lee et al., 1999). The C2 domain contains structures consistent with membrane association, and indeed does associate with phospholipid

membranes *in vivo* (Das et al., 2003). This membrane localization is thought to be important for activation of the protein and to enable the protein to interact with its membrane-bound substrates (Das et al., 2003).

Signaling and Regulation

PTEN, as mentioned earlier, can dephosphorylate tyrosine, serine, and threonine residues, as well as phosphorylated lipids. *In vitro*, PTEN has been shown to dephosphorylate FAK and Shc (Src and collagen homologue), which are proteins known to be important in motility and survival (Gu et al., 1999). However, the relative importance of this enzymatic function *in vivo* compared to its lipid phosphatase activity has been controversial (Yamada and Araki, 2001). Moreover, most research has focused on PTEN's ability to dephosphorylate lipids, in particular phosphatidyl inositol (3,4,5)-phosphate (PIP3), so this is where most of the information lies (Goberdhan and Wilson, 2003).

The primary biological function of PTEN is to antagonize phosphatidyl inositol 3-kinase (PI3-kinase) signaling, by converting PIP3 back to the PI3-kinase substrate PIP2 (Goberdhan and Wilson, 2003). Even though PIP3 acts a second messenger and has many substrates within the cell, PTEN expression has been found to mostly affect cellular processes regulated by the PIP3 downstream effector Akt (or PKB-protein kinase B) (Leslie and Downes, 2002). Akt has been shown to be a negative regulator of molecules that inhibit cell proliferation and survival (Leslie and Downes, 2002). However, the lack of PTEN has been shown to increase motility via Rac and Cdc42 (Liliental et al., 2000).

Clearly, PTEN is capable of regulating processes in primary tumorigenesis, as well as in invasion and metastasis.

PTEN activity is regulated by phosphorylation, localization, and transcription. When phosphorylated on serine and threonine residues in the C-terminal tail, PTEN is most likely monomeric and cytosolic (Leslie and Downes, 2004). The function of phosphorylation is controversial, but it appears to stabilize and reduce the activity of the protein (Leslie and Downes, 2002). In most cell types, PTEN appears to be largely cytosolic, thus requiring membrane localization to act on its downstream target PIP3 (Leslie and Downes, 2002). The C2 domain possesses intrinsic and significant membrane-binding potential, and this potential appears to be specific for acidic, phosphotidyl-inositol containing membranes (Maehama et al., 2004) (Leslie and Downes, 2004). The MAGI (membrane associated guanylate kinase with inverted orientation) proteins are also known to physically interact with PTEN via their PDZ domain, thereby resulting in an additional way that PTEN can be targeted to the membrane (Leslie and Downes, 2002).

1.6 Significance

The major cause of death from cancer is metastasis to the vital organs. Although it may be possible to eradicate a primary tumor by surgery or other therapeutic intervention, there is no effective therapy for advanced metastatic cancer. For breast cancer, the transition form a primary tumor to invasive cancer is estimated to average six

years, which provides ample time for therapeutic intervention (ACS 2005). Unfortunately, the mechanisms that underlie the malignant progression of this cancer are not very well understood, and there are very few proteins that have been identified as regulators of metastasis. Thus, understanding the progression of breast cancer to the metastatic state and the molecular changes that take place in malignant primary breast tumors are crucial for designing potential intervention strategies.

2. PTEN as a Negative Regulator of Human Breast Cancer Metastasis

2.1 Introduction

PTEN (phosphatase and tensin homologue deleted on chromosome ten) is a tumor suppressor gene that has been shown to be essential for normal cell development, but deleted or mutated during tumorigenesis in certain cancers and cancer predisposition syndromes (Bonneau and Longy, 2000; Dahia, 2000; Ali et al., 1999). Experimental mutational analysis of PTEN has been shown to result in unbalanced cell proliferation and cell survival (Vazquez and Sellers, 2000). In addition, it has been thought that the chromosome region where PTEN is located, region 10q22-24, includes one or more genes that play a role in several human malignancies (Dahia, 2000). PTEN deletions and mutations can occur during early stage transformation, or can be correlated with advanced cancer grade (Goberdhan and Wilson, 2003). With respect to endometrial and ovarian cancers, PTEN mutation tends to be found in the earlier stages (Vazquez and Sellers, 2000). Conversely, PTEN mutation is found to increase with respect to an increase in malignancy or higher grade tumors in glioblastoma and prostate tumors (Vazquez and Sellers, 2000). However, the degree to which PTEN can be used as a predictor of outcome is not known, and requires further study (Vazquez and Sellers, 2000).

Substrates of PTEN

PTEN is thought of as a dual specificity phosphatase, as it can dephosphorylate both protein and lipid substrates. One protein substrate shown to interact with PTEN is focal adhesion kinase (FAK). FAK is a key regulatory molecule in the processes of growth factor and integrin-stimulated cell motility and proliferation. In transformed cells and in analysis of human tumors, elevated FAK expression and activity have been correlated with progression to a malignant phenotype (Schlaepfer and Mitra, 2004). Studies have shown that PTEN interacts with FAK *in vitro* and causes dephosphorylation of its tyrosine residues (Gu et al., 1998; Tamura et al., 1998). Initially, PTEN regulation of FAK was an exciting and promising prospect in the investigation of cancer metastases, but this work has been irreproducible, and may be cell-line specific (Waite and Eng, 2002).

A more plausible substrate for PTEN is the lipid phosphoinositide (3,4,5)-trisphosphate (PIP3) (Dahia, 2000; Kandel and Hay, 1999; Stambolic et al., 1999; Tamura et al., 1999; Besson et al., 1999). The enzyme phosphoinositide 3-kinase (PI3-kinase) phosphorylates PIP(3,4)2 to yield PIP(3,4,5)3, thereby classifying PTEN as a PI3-kinase antagonist. PIP3 is a quantitatively minor phosphoinositide, rapidly and transiently produced in response to agonist stimulation. However, PIP3 acts as a critical second messenger molecule able to control the spatiotemporal organization of signaling pathways at the plasma membrane (Payrastre et al., 2001). Pathways regulated by PIP3 include cell survival and proliferation, cell motility and organization of the cytoskeleton, as well as glucose metabolism (Rameh and Cantley, 1999). Cells that lack PTEN are

unable to regulate the processes controlled by PIP3, which stimulates a variety of cellular phenotypes that favor oncogenesis and malignant progression (Sulis and Parsons, 2003).

PTEN in Motility and Invasion

PTEN has recently been implicated in the processes of motility and chemotaxis. Expressing ectopic PTEN in PTEN null cells inhibits cell migration (Tamura et al., 1998). Moreover, PTEN-null fibroblasts show enhanced rates of migration, which can be reduced by re-introduction of PTEN (Liliental et al., 2000). Originally, because PTEN had been shown to dephosphorylate FAK (focal adhesion kinase) *in vitro*, it was thought that this was the mechanism behind the regulation of cell motility by PTEN (Tamura et al., 1999). However, more research has shown that PTEN regulates motility by downregulation of the small GTPases Rac and Cdc42, via inactivation of PIP3 (Stiles et al., 2004; Liliental et al., 2000). Data from *Dictyostelium* has recently shown that localization and translocation of PTEN following a chemotractant stimulation is reciprocal to PI3-kinase location (Iijima et al., 2002) (Iijima and Devreotes, 2002). This model suggests that a chemotractant causes PI3-kinase to localize to the leading edge of the membrane, leading to PIP3 accumulation, Akt activation, and the formation of a pseudopod via PI3-kinase induced Rac activity. In parallel, PTEN dissociates from the leading edge of the membrane and relocates posteriorly, thus allowing more PIP3 to accumulate at the leading edge (Iijima et al., 2002; Iijima and Devreotes, 2002).

PTEN in Human Breast Cancer

In contrast to some cancers, only about 6-10% of breast cancers tested to date have inactivated PTEN (Li et al., 1997) (Cantley and Neel, 1999). In breast cancers, PTEN deletions do not play a dominant role in primary tumorigenesis according to PTEN mutational analysis of primary breast cancers (Perren et al., 1999). However, PTEN dysfunction may play a role in advanced breast cancer, as was shown in more invasive breast carcinomas (Bose et al., 2002). The loss of PTEN has been shown to predict resistance to chemotherapeutic treatment in breast cancer (Pandolfi, 2004). Moreover, reduced PTEN expression has been associated with poor outcome and angiogenesis in invasive ductal carcinoma of the breast (Lee et al., 2004). Additionally, reduced PTEN expression predicts relapse in patients with breast carcinoma treated by tamoxifen (Shoman et al., 2005). Clearly, there is a link between PTEN expression and advanced breast cancers. Exploration into the mechanisms by which PTEN affects breast cancer is crucial to understanding and preventing further mortality. Therefore, the role of PTEN in malignant breast cancer requires further investigation.

We hypothesize that due to its decreasing expression in invasive breast cancer tissue and its central role in regulating cellular motility, PTEN is a negative regulator of metastasis in human breast cancer. To test this hypothesis, we investigated endogenous PTEN and PI3-kinase expression in a range of human breast cancer cells. We localized PTEN both prior and subsequent to stimulation, and colocalized the protein with proposed downstream effectors involved in motility signaling pathways. Finally, we show that exogenous PTEN expression negatively modulates cell motility in invasive,

metastatic human breast cancer cells. Taken together, these data represent an investigation into the role of PTEN and its downstream effectors in the invasive capabilities of human breast cancer.

2.2 Materials and Methods

Cell Culture

Metastatic human breast cancer cell lines T47D, HS578t, MDA-MB-231 and MDA-MB-435 were cultured in supplemented minimum essential medium (Gibco™, CA) with 10% fetal bovine serum (Tissue Culture Biologicals, CA) and incubated in a humidified 5% CO_2 atmosphere at 37°C.

Immunoblotting

Proteins from total cell lysate were separated by 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and probed with a mouse monoclonal anti-PTEN antibody (Cell Signaling, MA) or a rabbit polyclonal anti-PI3 kinase p85 subunit alpha antibody (Santa Cruz, CA). Immunoblots were detected subsequent to incubation with an HRP-conjugated secondary antibody (Pierce Endogen, IL) with the SuperSignal West Femto-Substrate chemiluminescence kit (Pierce Endogen, IL) and Kodak Biomax MR film (Fisher Scientific, TX).

Stimulation and Immunostaining of Breast Cancer Cells

Cells were cultured on coverslips, and starved for 24 to 48 hours prior to stimulation with Heregulin (Neomarkers, CA). Cells were fixed subsequent to stimulation with 4% paraformaldehyde (Sigma Chemical Corp., MO), permeabilized with 0.5% Triton X-100 (Sigma, MO), and blocked with 5% goat serum (Gibco^{TM}, CA) and 5% BSA (Sigma Chemical Corp., MO) in 1XPBS. To visualize localized PTEN, cells were incubated with anti-PTEN antibody (Cell Signaling, MA) followed by a secondary antibody conjugated to FITC (Pierce, IL). Cells were imaged using an Olympus upright fluorescence microscope, and digital pictures were taken with Spot Advanced digital camera software (Diagnostic Instruments Inc., MI).

Exogenous PTEN Expression and PIP3 staining

GFPPTEN vector (kind gift of Kenneth Yamada, NIH) was transfected into the MDA-MB-231 cell line using Lipofectamine Plus Reagent (Gibco^{TM}, CA). Maximal expression was achieved 24-48 hours post transfection. PTEN was introduced into MDA-MB-435 cell line via the Rev Tet-Off stable gene expression system (Clontech, CA). Briefly, cells were retrovirally transfected with the plasmid encoding the Tet-Off regulatory protein and selected until stable. Cells were then retrovirally transfected with the plasmid containing the gene of interest fused to the Tet response element. Cells expressing both plasmids were then selected by drug selection (puromycin resistance). Gene induction was achieved by removing the repressing antibiotic, doxycycline.

Subsequent to PTEN expression, cells were fixed and stained with a mouse monoclonal antibody to PIP3 (Echelon, UT) followed by an IgM secondary antibody conjugated to FITC (Pierce, IL).

Rac Activity Assay

Rac activity assays were performed as described in (Benard et al., 1999) with minor modifications. Briefly, cells were washed twice with 1X PBS, lysed with 1X ice cold lysis buffer, and scraped from the plate. Lysates were then incubated at 4° for 1 hour with 10μg of PAK-PBD Protein GST Beads (Cytoskeleton Inc., CO). The bead pellet was then washed once with wash buffer containing 1% Nonidet P-40 (Calbiochem, CA) and twice without Nonidet P-40. The bead pellet was finally suspended in 20 μl Laemelli sample buffer. Proteins from total cell lysate, as well as the bead pellet, were separated by 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and blotted using a monoclonal anti-Rac (clone 32A8) antibody (Upstate Biotechnology, NY). Immunoblots were detected with the SuperSignal West Femto-Substrate chemiluminescence kit (Pierce Endogen, IL) and Kodak Biomax MR film (Fisher Scientific, TX).

Wound Healing Assay

Cells were cultured on coverslips until 100% confluency, then transfected with GFPPTEN vector. 36 hours post transfection, cells were again confluent and were stimulated by wounding as described in (Nobes and Hall, 1999). Wound healing assays

were performed by wounding a confluent monolayer of cells with a sterile 21G11/2 Precision Glide™ needle (Becton Dickinson and Co., NJ). Cells were fixed with 4% formaldehyde (Sigma Chemical Corp., MO) six hours subsequent to wounding. To visualize actin cytoskeleton, cells were stained with rhodamine phalloidin (Molecular Probes Inc., OR). Cells were imaged using an Olympus upright fluorescence microscope, then photographed with Spot Advanced digital camera software (Diagnostic Instruments Inc., MI).

Apoptosis Assay

Cells were plated on coverslips, transiently transfected with GFPPTEN and assayed for cell death 24, 36, and 48 hours post transfection. Briefly, cells were fixed and permeablized in ice-cold 70% ethanol and incubated with RNase A (Sigma, MO) and propidium iodide (Sigma, MO) at 37 degrees for 30 minutes. Under the Olympus upright fluorescent scope, the areas of control cell nuclei (Lipofectamine alone) as well as those cells expressing GFPPTEN, were traced and calculated using Spot Advanced digital camera software (Diagnostic Instruments Inc., MI).

2.3 Results

PTEN expression decreases as metastatic efficiency increases in human breast cancer cells.

Although similar data was subsequently published by others,

(Chung et al., 2004; Bose et al., 2002), a direct correlation was found between breast cancer progression and PTEN expression (Figure 2.1). The T47D breast cancer cell line is derived from a primary human breast tumor, is not invasive in *in vitro* studies, and expresses a relatively high amount of endogenous PTEN. The HS578T and MDA-MB-231 breast cancer cell lines are low metastatic, invasive, and express some endogenous PTEN. The MDA-MB-435 cell line is a highly metastatic, highly invasive cell line that expresses almost no endogenous PTEN. Because of this result, we were encouraged to investigate PTEN as a regulator of metastasis, and not primary tumorigenesis, in human breast cancer.

Additionally, the expression of PI3-kinase was found to be directly correlated with breast cancer progression: PI3-kinase expression decreased with decreased metastatic progression (Figure 2. 1). Recent studies have suggested that PTEN and PI3-kinase exhibit a reciprocal relationship with respect to motility in *Dictyostelium* (Funamoto et al., 2002). Our data shows that those cells exhibiting the more invasive phenotype have lower levels of endogenous PTEN and higher levels of PI3-kinase, and those exhibiting the less invasive phenotype have higher levels of PTEN and lower levels of PI3-kinase. This observation supports the idea that PTEN and PI3-kinase exhibit a reciprocal relationship in regulation in human breast cancer: PTEN acts as a PI3-kinase antagonist in a panel of human breast cancer cells.

PTEN translocates away from the leading edge the cell upon stimulation.

To understand the role of PTEN in breast cancer cells following PI3-kinase activation, we investigated the subcellular localization of PTEN following heregulin stimulation in MDA-MB-231 breast cancer cells. In the low metastatic cell line MDA-MB-231, endogenous PTEN localizes to the leading edge of quiescent cells (Figure 2.2). However, upon stimulation with the growth factor heregulin, which stimulates EbrB2/Her2 receptors to stimulate PI3-kinase, PTEN appears to move away from the leading edge of the cell. Because of this result, we hypothesized that PTEN was acting as a negative regulator of PIP3 at the membrane of the leading edge in resting cells. At rest, PTEN is localized uniformly at the membrane, inactivating PIP3 subsequent motility signals. Upon stimulation, PTEN retreats from the leading edge, allowing PI3-kinase to activate PIP3 and classical chemotactic responses. This is currently the working model for PI3-kinase/PTEN mediated chemotaxis (Funamoto et al., 2002).

PTEN localizes with focal adhesions in breast cancer cells under normal serum conditions.

Focal adhesions are sites of cell-matrix adhesion and are essential to understanding the process of cell motility. They represent highly regulated signaling scaffolds, which signal for motility, survival, as well as proliferation (Carragher and Frame, 2004). Many key proteins involved in migratory pathways have been localized to focal adhesions, including FAK and PI3-kinase (Brunton et al., 2004). Our data localizes PTEN to the same cellular regions in which focal adhesions are found in metastatic

human breast cancer cells while under normal tissue culture conditions in serum (Figure 2.3). This observation again implicates PTEN in the regulation of motility and invasion in human breast cancer.

PTEN colocalizes with FAK in breast cancer cells under normal serum conditions.

Not only does our data place PTEN at focal adhesions, but we can also colocalize PTEN with FAK (Figure 2.4). FAK is a versatile protein known to participate in a myriad of signaling cascades (Mitra et al., 2005). Not only can FAK activate the small GTPases Rac and Cdc42 to result in active motility, but activated FAK can also protect the cell from apoptosis and activate MAP kinase signaling (Schaller, 2001). PTEN has been shown to have the ability to dephosphorylate, and subsequently deactivate, FAK *in vitro* (Tamura et al., 1999). Deactivation of FAK could have severe consequences in the cell, including inhibition of motility, as well as programmed cell death (Schaller, 2001). Because we can place PTEN in very close proximity to FAK, and FAK has been shown to be a substrate of PTEN, we propose that PTEN is directly dephosphorylating FAK *in vivo*, in human breast cancer cells.

Ectopic PTEN expression reduces PIP3 expression in MDA-MB-231 and MDA-MB-435 breast cancer cell lines.

PIP3 has been shown to be a substrate of PTEN both *in vitro* and *in vivo* (Sulis and Parsons, 2003). PTEN dephosphorylates PIP3 at the D3 position of the inositol ring, effectively antagonizing PI3-kinase activity. We show a reduction in PIP3 levels in both

an invasive, highly metastatic cell line (MDA-MB-435) with low endogenous PTEN expression, and an invasive, low metastatic (MDA-MB-231) cell line with endogenous PTEN expression subsequent to the ectopic expression of PTEN (Figures 2.5 and 2.6). Figure 2.5 demonstrates the decrease in PIP3 in response to PTEN expression in MDA-MB-231 breast cancer cells. Those cells expressing GFP-tagged PTEN exhibit a marked difference in their PIP3 expression levels compared to the non-transfected controls. Because PTEN is a tumor suppressor, and negatively regulates pathways of survival and proliferation, ectopic PTEN expression has been found to cause apoptosis is several types of cells (Parsons and Simpson, 2003). However, apoptosis is not occurring up to 48 hours post transfection in MDA-MB-231 cells, therefore, the downregulation of PIP3 observed in our studies is not due to cellular apoptosis (Figure 2.6). Figure 2.7 demonstrates a decrease in PIP3 expression in response to ectopic PTEN expression in MDA-MB-435 breast cancer cells, without significant apoptosis (Figure 2.8). Additionally, it has been previously reported that ectopic PTEN expression results in a downregulation of Rac in fibroblasts (Liliental et al., 2000). However, no such downregulation was found in MDA-MB-435 cells (Figure 2.9).

Furthermore, the endogenous PIP3 expressed in MDA-MB-231 appears to be lesser of that expressed in MDA-MB-435 (Figure 2.5, right panel, Figure 2.7b, left panel). This effect is presumably due to the endogenous PTEN levels exhibited in these cells. Evidence to support this conclusion is present in Figure 2.1. Figure 2.1 shows endogenous PI3-kinase expression as well as endogenous PTEN. MDA-MB-231 appears

to exhibit higher levels of PI3K than MDA-MB-435. Therefore, higher levels of PIP3 found in MDA-MB-435 could not be a result of elevated PI3-kinase levels.

Ectopic PTEN expression negatively regulates migration in metastatic human breast cancer cells without causing apoptosis.

Subsequent to stimulation in a wound healing assay, cells expressing ectopic PTEN fail to polarize and migrate as compared to control cells (Figure 2.10). Control cells exhibit a motile and polarized phenotype as demonstrated with actin staining (left panels), migrating toward the stimulus. Cells not expressing ectopic PTEN form lamellipodia towards the stimulus, a process which is a hallmark of both motility and invasion. Conversely, those cells expressing the GFP-tagged PTEN construct (right panels) exhibit no polarization or motility toward the stimulus. These cells form no lamellipodia towards the wound edge, which is indicative of a stationary cell. GFPPTEN expression does not cause apoptosis in cells up to 48 hours post transfection, so the resulting phenotype cannot be due to the stimulation of apoptotic pathways (Figure 2.6).

2.4 Discussion

PTEN is a tumor suppressor in several types of cancer, including prostate cancer , endometrial cancer, and glioblastoma. However, PTEN is missing in only 11% of *in situ* breast cancers, compared to 38% of invasive cancers (Bose et al., 2002). Data presented here show that decreasing PTEN expression directly correlates with increasing invasive

and metastatic potential of breast cancer cell lines. Therefore, we formulated the working hypothesis is that PTEN acts as a negative regulator of metastatic progression in human breast cancer. The eventual aim of this research was to test the innovative concept of PTEN acting as a metastasis suppressor in breast cancer.

PTEN and the PI3-kinase Product, PIP3

First, we demonstrated that PTEN retreats from the leading edge of the cell upon stimulation with growth factor (heregulin). We hypothesized that this was a reciprocal relationship with PI3-kinase, allowing PI3-kinase to phosphorylate PIP2 to yield PIP3 upon stimulation. This is now the current model for the regulation of cellular motility during chemotactic responses (Funamoto et al., 2002). Presumably, endogenous PTEN negatively regulates motility by being active at the leading edge. At the leading edge, PTEN downregulates the amount of PIP3, which signals to the Rho GTPases to induce cellular motility. Subsequent to stimulation, PTEN moves away from the leading edge, allowing PIP3 to accumulate and activate downstream signals (Funamoto et al., 2002).

Additionally, ectopic PTEN expression was shown to reduce endogenous PIP3 levels in two different human metastatic breast cancer cell lines without causing the cells to undergo apoptosis. Ideally, cells with less endogenous PTEN exhibit enhanced migratory abilities due to the upregulation of PIP3 and subsequent Rho GTPase activation (Liliental et al., 2000). To support this, we show that ectopic PTEN reduces the ability of the MDA-MB-231 cell line to react effectively to stimulus. Therefore, the model becomes obvious: PTEN downregulates PIP3, which negatively regulates motility

pathways. However, ectopic PTEN expression has no effect on Rac activation in MDA-MB-435 cells. Classically, it has been assumed that PIP3 activates motility via the small GTPases due to the ability of PIP3 to activate guanine nucleotide exchange factors (GEFs) that are known to activate Rho, Rac, and Cdc42 (Rameh and Cantley, 1999). Perhaps other motility pathways are being downregulated instead. Akt, or PKB, is itself an oncogene that acts downstream of PIP3 to activate cell survival (Toker, 2000). Recently, Akt has been shown to activate motility pathways, as well as cell survival pathways, in U87MG human glioma cells (Kim et al., 2005; Pu et al., 2004). Furthermore, Akt activation has been correlated with tumor invasion and oncogene expression in thyroid cancer (Vasko et al., 2004). Another downstream effector of PIP3 is PKC (protein kinase C) (Rameh and Cantley, 1999). Classically, PKC activation by PIP3 was thought to activate cell proliferation (Rameh and Cantley, 1999). However, recent evidence has shown that PKC could be involved in cell motility via the production of certain metalloproteinases (Urtreger et al., 2005). Clearly, Rac is not the only protein activated by PIP3 that could increase cell motility.

PTEN and its Protein Substrate, FAK

Additionally, PTEN was shown to localize to focal adhesion and colocalize with FAK in human breast cancer cells. Focal adhesions are multimolecular complexes of signaling scaffolds and structural proteins, and it is possible that PTEN could be acting at these sites. FAK, a protein often used as a marker for focal adhesion sites, has been shown to be a substrate of PTEN (Tamura et al., 1999). It would be easy to explain the

effect PTEN has on motility by assuming that PTEN does directly deactivate FAK, and we present good evidence to support this model. Furthermore, researchers have reported a clear PTEN/FAK relationship in their model systems (Zhang et al., 2003; Zhang et al., 2004; Gautam et al., 2003). However, the experiments in which FAK was found to be a direct substrate of PTEN are somewhat controversial, and have been unable to be repeated by other groups (Yamada and Araki, 2001). Because of this, most research has focused on the lipid-phosphatase activity of PTEN in contrast to its protein phosphatase activity.

It is possible that colocalization does not necessarily mean that PTEN is directly dephosphorylating FAK. PTEN is admittedly found at the leading edge of migrating cells, and so is FAK, when present in focal adhesions (Webb et al., 2004). PI3-kinase is also found at the leading edge in migrating cells (Chung and Firtel, 2002). It is possible that PTEN is directly dephosphorylating PIP3, which is initiating a cascade of signals that result in the negative regulation of motility. A negative regulation of motility is most likely going to affect FAK, whether direct or indirectly, because of the central role FAK plays in regulating motility. Clearly, more experiments are needed to show a direct dephosphorylation of FAK by PTEN in human breast cancer.

Conclusion

In conclusion, the data presented here would be much stronger with additional experiments. More precisely, the data would be much stronger with a stable cell line. Because PTEN has been shown to cause apoptosis in cells, and the nude mouse model of

experimental metastasis was proposed to show the efficacy of PTEN as a bona fide metastasis suppressor, the Tet-Off repressor system was used to make the stable cell lines expressing ectopic PTEN. Efforts to produce this stable line with Clontech's Tet-Off Repressor System were unsuccessful, but not without a brief period of success (refer to figure 2.7). Taken together, promising preliminary data was achieved, but the project eventually failed due to the lack of a permanent stable cell line.

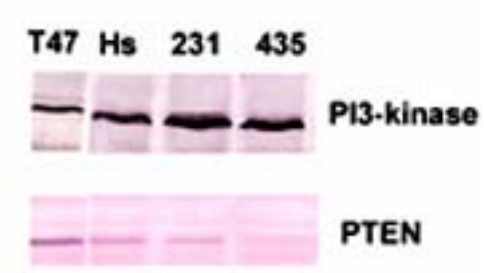


Figure 2.1. Expression of PI3-kinase and PTEN in breast cancer cell lines. Whole cell lysates of the non-metastatic breast cancer cell lines T47D and Hs578T and the metastatic breast cancer cell lines MB-231 and MB-435 were subjected to SDS-PAGE followed by western blotting for PI3-kinase using an anti-p85 antibody (top panel) and PTEN using an anti-PTEN antibody (bottom panel). Equal loading of lanes was maintained by using the same amount of total protein/lane.

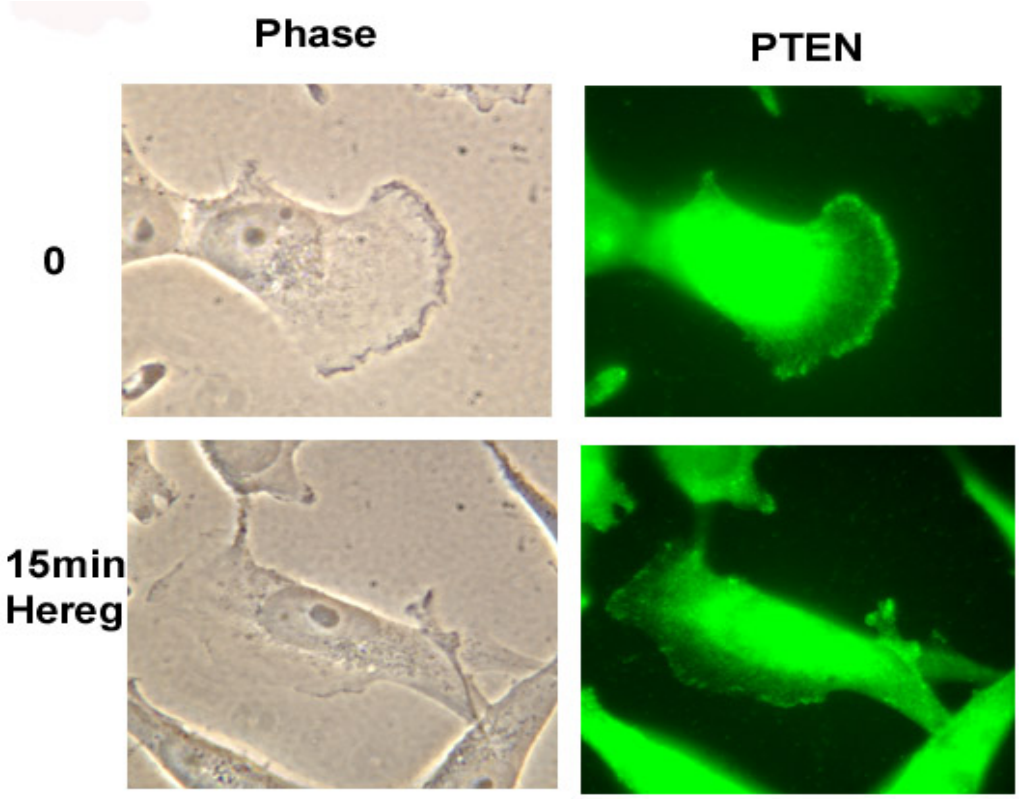


Figure 2.2. PTEN distribution in response to stimulation. MDA-MB-231 cells were plated on coverslips, and either serum starved (upper panel) or starved and then stimulated for 15 minutes with heregulin (lower panel). Cells were then fixed and subjected to immunocytochemistry with an antibody to PTEN followed by a secondary antibody conjugated to FITC.

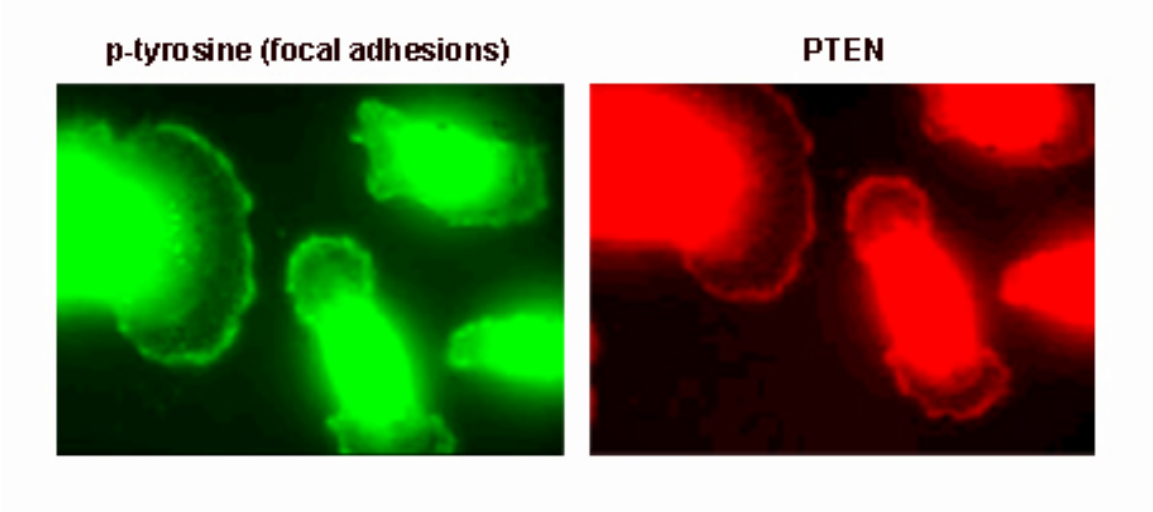


Figure 2.3. Subcellular localization of PTEN and focal adhesions. MDA-MB-231 cells were plated on coverslips and immunostained for focal adhesions with a monoclonal anti-phosphotyrosine antibody and PTEN with a polyclonal anti-PTEN antibody. Anti-phosphotyrosine incubation was followed by a mouse secondary antibody conjugated to FITC; anti-PTEN incubation was followed by a rabbit secondary antibody conjugated to rhodamine. Pictures represent the same microscopic field under different fluorescent filters.

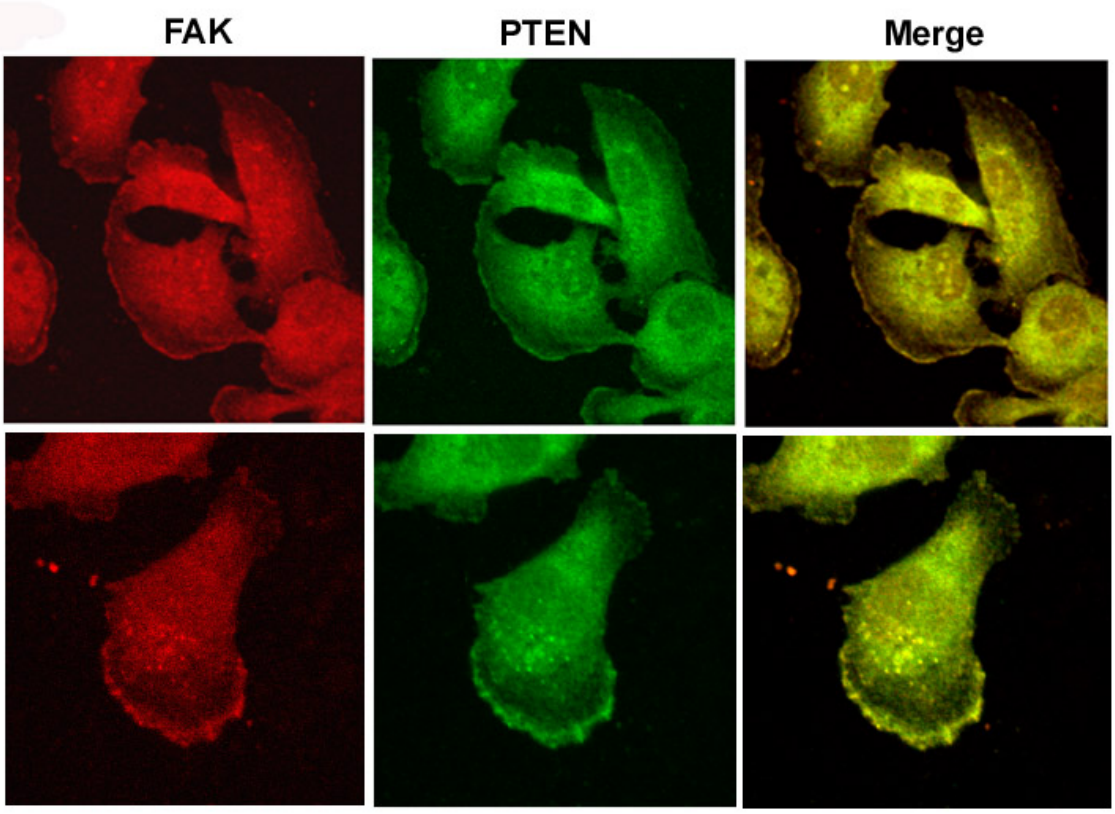


Figure 2.4. Localization of FAK and PTEN in MDA-MB-231 breast cancer cells. Cells were plated on coverslips, fixed, and immunostained for FAK and PTEN. To localize FAK, a mouse monoclonal anti-FAK antibody was used, followed by an anti-mouse secondary antibody conjugated to FITC. To localize PTEN, a rabbit polyclonal anti-PTEN antibody was used, followed by an anti-rabbit secondary antibody conjugated to rhodamine. Rows represent the same microscopic fields, columns represent different fluorescent fields. The right-most column represents an overlay using Adobe Photoshop software.

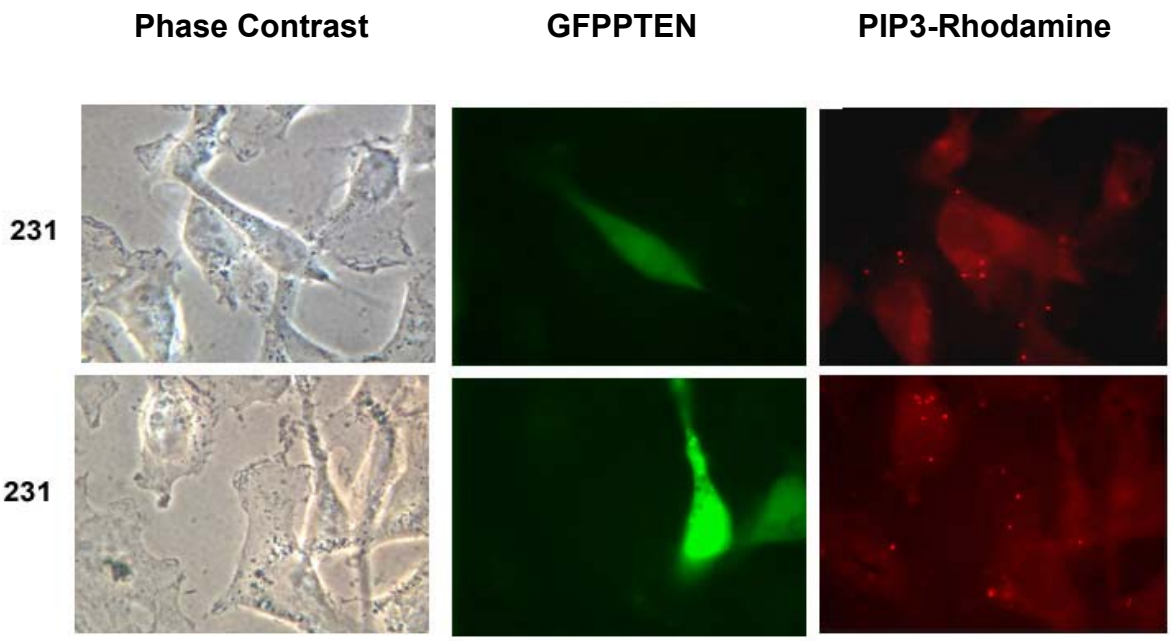


Figure 2.5. Ectopic PTEN expression and endogenous PIP3 expression. MDA-MB-231 cells were transiently transfected with GFP-tagged PTEN construct. Cells were then fixed and stained with an antibody to PIP3, followed by rhodamine conjugated secondary antibody. Upper and lower rows represent the same microscopic field, left column is DIC, middle column is GFP (PTEN) visualized with an FITC filter, right most panel is rhodamine (PIP3), visualized with a rhodamine filter.

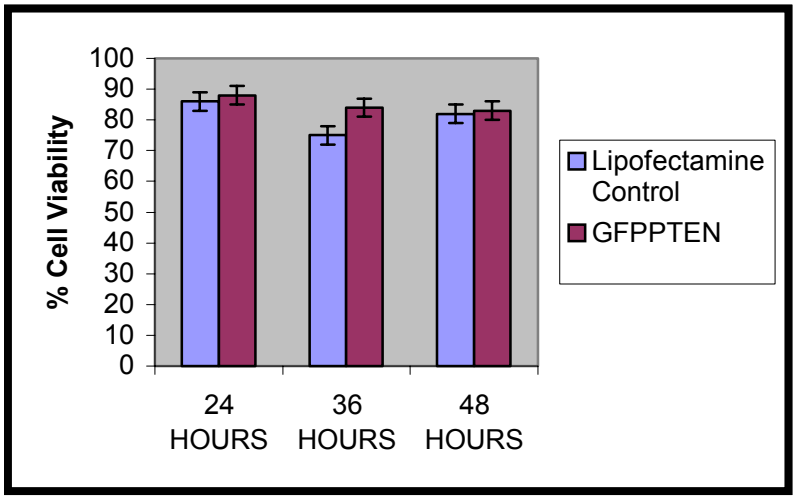


Figure 2.6. Viability of MDA-MB-231 breast cancer cells subsequent to ectopic PTEN expression. Apoptosis assays were performed by staining the nuclei of MDA-MB-231 cells with propidium iodide 24, 36, and 48 hours post-transfection with GFPPTEN and Lipofectamine reagent.

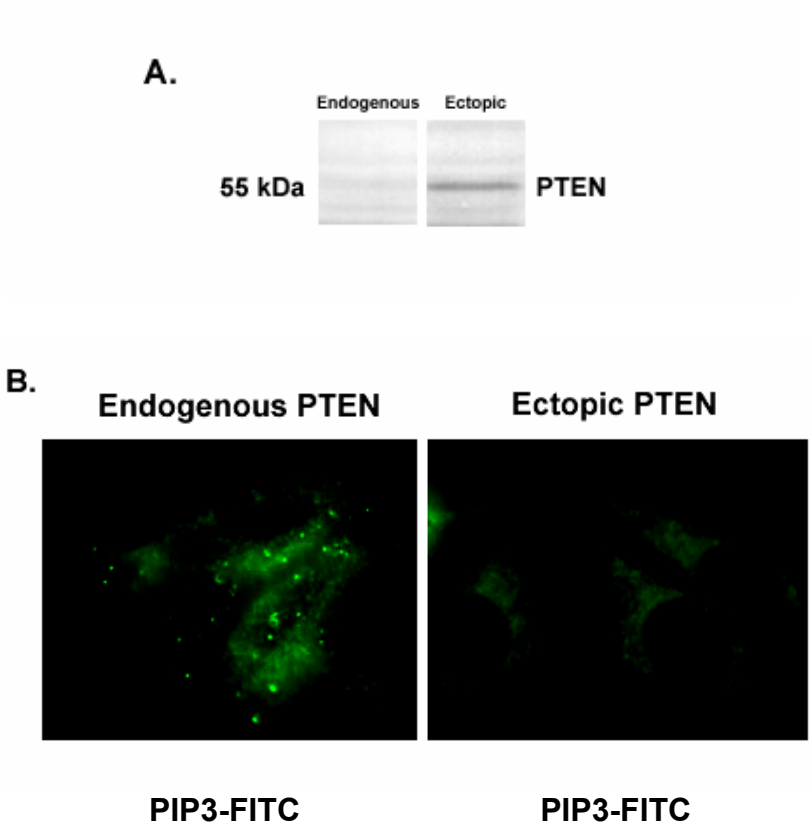


Figure 2.7. Endogenous PIP3 levels and ectopic PTEN expression. MDA-MB-435 cells were stably transfected with the Tet-Off repressor system from Clontech, expressing either control vector or PTEN. 24 hours post removal of doxycycline, PTEN expression was determined by western blot with an anti-PTEN antibody **(a)** and PIP3 expression was determined by immunofluorescence using an anti-PIP3 antibody followed by a secondary antibody conjugated to FITC **(b)**.

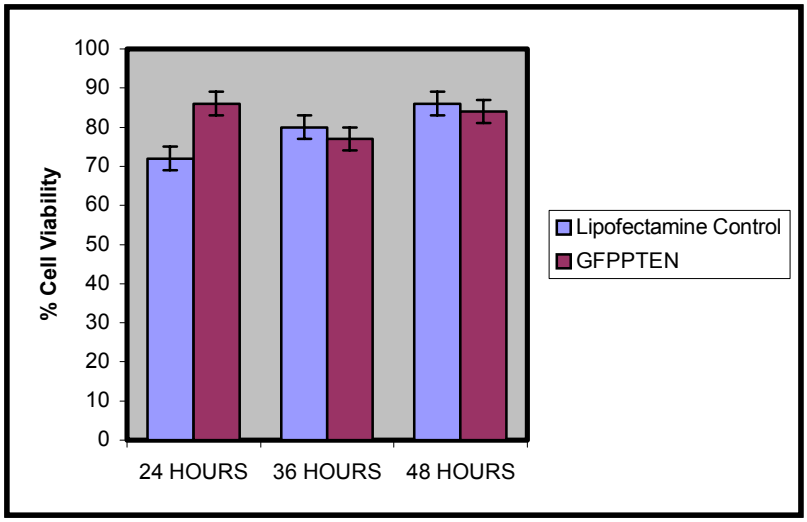


Figure 2.8. Viability of MDA-MB-435 cells subsequent to ectopic PTEN expression. Apoptosis assays were performed by staining the nuclei of MDA-MB-435 cells with propidium iodide 24, 36, and 48 hours post-transfection with GFPPTEN and Lipofectamine reagent.

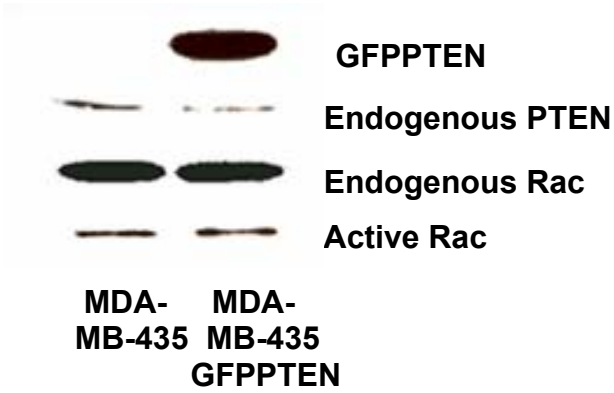


Figure 2.9. Rac activity levels subsequent to ectopic PTEN expression. Whole cell lysates of MDA-MB-435 cells ectopically expressing GFPPTEN were subjected to immunoblot for GFPPTEN, endogenous PTEN, and endogenous Rac expression. Cells were also assayed for activated Rac subsequent to control or PTEN expression.

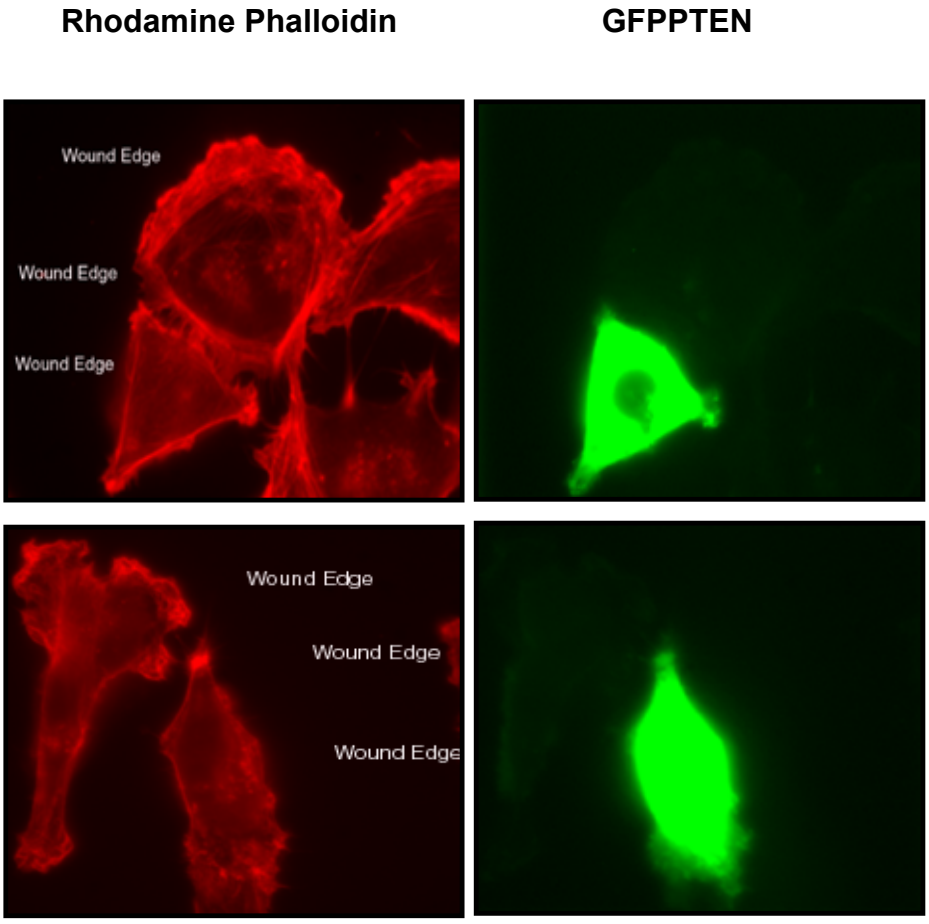


Fig 2.10. Ectopic PTEN expression curtails migration. GFPPTEN (kind gift of Kenneth Yamada of the NIH) was transfected into a confluent monolayer of MDA-MB-231 cells. 36 hours post-transfection cells were wounded, allowed to migrate, fixed and stained with rhodamine phalloidin. Left column, cells stained with rhodamine phalloidin. Right column, same cells visualized with FITC filter to visualize GFP-fluorescence. White lettering in the left panels represents the area where the wound was made (wound edge).

3. Characterization of the Metastatic Panel of MDA-MB-435 Variants

3.1 Introduction

To undergo metastatic transformation, cells must sense signals that inform them to actively migrate through the three-dimensional network of the proteins of the extracellular matrix (ECM). When epithelial cells, such as mammary cells, undergo metastatic transformation, they must migrate through the basement membrane, which is a type of ECM that is organized in to thin, specialized sheets. Hallmarks of this process include invasive morphology, migratory phenotype, and hyperactivation or overexpression of the proteins that regulate these processes. Such proteins include the integrin family of transmembrane receptors as well as the Rho family of small GTPases, Rho, Rac, and Cdc42.

Integrins in Metastasis

Integrins are heteromeric, transmembrane glycoproteins that serve as the interface between the actin cytoskeleton and the proteins of the extracellular matrix (ECM). These heterodimers contain an α and a β subunit, both of which make important contributions to various aspects of overall integrin function. Upon activation, the integrin heterodimers cluster into specialized adhesive structures, focal adhesions (FAs) and focal contacts (FACs), in which numerous structural and signaling components are concentrated

(Schoenwaelder and Burridge, 1999; Hynes, 2002; Martin et al., 2002). During metastatic progression, cancer cells undergo constant interaction with their immediate environment via these focal adhesion contacts, resulting in a myriad of integrin-mediated signaling cascades (Mercurio et al., 2001; Petit and Thiery, 2000; Petit and Thiery, 2000). Aberrant integrin expression, and the subsequent disregulation of FACs, has been implicated in the progression of tumor invasion and the process of metastasis (Brakebusch et al., 2002; Kassis et al., 2001). Specifically, higher expression of α6 integrin was associated with the metastatic phenotype and malignant progression of breast cancer cells (Mukhopadhyay et al., 1999; Shimizu et al., 2002). In addition, high expression level of α6 in human breast carcinoma has been correlated with tumor progression and poor prognosis (Friedrichs et al., 1995; Tagliabue et al., 1998).

α6β1 Integrin in Metastatic Progression

The α6 integrin dimerizes with either β4 or β1 to bind laminin, the major constituent of the basement membrane (Hintermann and Quaranta, 2004). Because MDA-MB-435 cells do not express the β4 integrin, motility in this cell line is mainly associated with α6β1 integrin (Wewer et al., 1997; Mukhopadhyay et al., 1999). Many studies have demonstrated a critical role for the β1 integrin in cell migration, invasion, and supramolecular assembly of extracellular matrix proteins (Brakebusch et al., 1997; Sakai et al., 1998). Studies have shown that cells lacking the beta1 integrin subunit have poor directed cell migration to platelet-derived growth factor or epidermal growth factor, ligands of receptor tyrosine kinases (Sakai et al., 1998). Additionally, β1 integrin has

shown to be overexpressed in certain invasive cancers, and is required for the invasive behavior of these cells (Brockbank et al., 2005). To explain this link between integrin β1 and invasion, studies have shown that integrin β1 is capable of regulating members of the Rho family of small GTPases (Gimond et al., 1999; Miao et al., 2002; Hirsch et al., 2002; Sturge et al., 2002).

Rho Family Proteins in Metastasis

The activation of the Rho family of small GTPases, namely Rac, Rho, and Cdc42, is a critical event in the integrin-mediated regulation of the cellular processes of adhesion, migration, and invasion (Miranti and Brugge, 2002; Hynes, 2002). All Rho GTPases have been implicated in the turnover of FACs, a critical step in cell motility. Subsequent to activation, Rho GTPases interact with downstream target proteins to induce specific cellular responses: Rac regulates the polymerization of actin at the cell periphery to produce lamellipodia, Rho regulates cell contractility and the assembly of actin stress fibers, while activated Cdc42 induces the formation of filopodia (Hall and Nobes, 2000). However, during the processes of adhesion, migration, and invasion, crosstalk between the Rho GTPases, their isoforms, and their downstream effectors are coordinated in a highly complex and not completely understood manner (Schmitz et al., 2000). Activation of appropriate levels, together with temporal and spatial coordination, must be precisely regulated in order to achieve normal adhesion and motility (Price and Collard, 2001). The balance between Rac, Cdc42, and Rho, as well as the localized activity of these

proteins, is essential for the determination of cellular morphology and invasive behavior (Evers et al., 2000).

Integrin signaling subsequent to clustering and activation includes the tyrosine phosphorylation and activation of the Epidermal Growth Factor Receptor (EGFR), a common upstream effector of the Rho family GTPases, usually activated by the EGF (epidermal growth factor) ligand (Moro et al., 1998; Miyamoto et al., 1996). In fact, Rac1 has been shown to be required for the EGF-induced migration of breast carcinoma cells (O'Connor and Mercurio, 2001). Moreover, overexpression of the EGFR in breast cancer cells has been shown to increase invasiveness and metastasis, via Rac1 and Cdc42 (Sturge et al., 2002). However, evidence shows that the contribution of the Rac1 and Cdc42 proteins to tumor cell invasion in breast cancer is not due to genetic mutation (Fritz et al., 2002). Changes in the activity levels of these proteins due to upregulation of upstream activators instead has been shown to be responsible for the promotion of tumor cell invasiveness (Price and Collard, 2001; Fritz et al., 2002). Conversely, the overexpression of the RhoC gene has been strongly implicated in tumor progression, and has been shown to result in a motile and invasive phenotype when overexpressed in human mammary epithelial cells (Clark et al., 2000; Kleer et al., 2002).

To understand the role of Rho GTPases and their correlation to integrin expression in metastatic breast cancer, we used isolated variants of the MDA-MB-435 metastatic breast cancer cell line according to integrin α6 expression and metastatic efficiency in the mouse model of experimental metastasis (Mukhopadhyay et al., 1999). Data presented here shows that increased α6 integrin protein expression and increased

migratory ability toward reconstituted proteins of the basement membrane does correlate with increasing metastatic potential. Moreover, increased Rho and Rac (but not Cdc42) activity, as well as increased RhoC protein expression, correlates with an increased metastatic morphology and phenotype. Together, these data suggest that increased expression of the α6β1 integrin heterodimer contributes to the metastatic phenotype of MDA-MB-435 breast cancer cell variants via its effects, direct or indirect, on the activity of the small GTPases Rac and Rho.

3.2 Materials and Methods

Cell Culture

Variants of the human breast cancer cell line MDA-MB-435, a kind gift from Dr. Janet Price (MD Anderson Cancer Center, Houston, TX), were selected according to α6 expression and metastatic efficiency in the nude mouse model as described in (Mukhopadhyay et al., 1999). Cells were cultured in supplemented minimum essential medium (Gibco^{TM}, CA) with 10% fetal bovine serum (Tissue Culture Biologicals, CA), and cultured in a humidified 5% CO_2 atmosphere at 37°C.

Immunoblotting

Proteins from total cell lysate were separated by 12% SDS-PAGE gel, transferred to a nitrocellulose membrane, and probed with a goat polyclonal anti-a6 integrin antibody. (Santa Cruz Biotech, CA). Immunoblots were detected with the SuperSignal

West Femto-Substrate chemiluminescence kit (Pierce Endogen, IL) and Kodak Biomax MR film (Fisher Scientific, TX).

Wound Healing Assay

Cells were cultured on coverslips until 100% confluency, then stimulated by wounding as described in (Nobes and Hall, 1999). Wound healing assays were performed by wounding a confluent monolayer of cells with a sterile 21G11/2 Precision Glide™ needle (Becton Dickinson and Co., NJ).

Immunofluorescence Microscopy

Cells were cultured on coverslips either until 50% confluency, or until 100% confluency. Cells were either fixed at 50% confluency, or two hours after wounding using 4% formaldehyde (Sigma Chemical Corp., MO). Cells were then permeabilized with 0.5% Triton X-100 (Sigma, MO), and blocked with 5% goat serum (Gibco™, CA) and 5% BSA (Sigma Chemical Corp., MO) in PBS. To visualize F-actin, cells were stained with rhodamine phalloidin (Molecular Probes Inc., OR), and a mouse monoclonal anti-phosphotyrosine antibody, clone 4G10 (Upstate Biotechnology, NY), followed by FITC-conjugated goat anti mouse IgG (ICN Biomedicals Inc., CA) to visualize the focal adhesions. Cells were imaged using an Olympus upright fluorescence microscope, then overlayed with Spot Advanced digital camera software (Diagnostic Instruments Inc., MI).

Constructs and Transfections

Rac1 mutant cDNA (myc-Rac1(T17N)) and Cdc42 mutant cDNA (myc-Cdc42(T17N)) were generous gifts from Dr. Gary Bokoch of the Scripps Institute (La Jolla, CA). Rac3 mutant cDNA (myc-Rac3(T17N)) was a generous gift from Dr. Ulla Knaus of the Scripps Institute (La Jolla, CA). Mutant Rac and Cdc42 mutant cDNAs were digested out of the pRK5myc vector and inserted in to the multiple cloning site of the pIRESneo2 vector (Clontech, CA).

PIRESneo2 vector alone, or vectors encoding myc-tagged Rac1(T17N) or Cdc42(T17N) were transfected into cell variants using Lipofectamine Plus Reagent (Gibco^{TM}, CA). Maximal expression was achieved 24-48 hours post transfection.

Haptotaxis Migration Assays

Cell migration and invasion assays were performed as described in (Klemke et al., 1998). Briefly, modified Boyden chambers (tissue culture treated, 6.5 mm diameter, 10μm thickness, 8 μm pores, Transwell®, Costar Corp., Cambridge, MA) were coated on the underside, of the membrane with matrigel (Fisher Scientific, TX), or 50 μg/ml laminin (Gibco BRL, MD) overnight at 4° and then placed into a trans-well. Serum starved cells (10^5 cells) were added to the upper surface of each migration chamber and allowed to migrate to the underside of the membrane for 4 hours. The non-migratory cells on the upper membrane surface were removed with a cotton swab, and the migratory cells attached to the bottom surface of the membrane stained with propidium iodide (PI) (CalBioChem-Novabiochem Corp., CA). Briefly, cell were fixed and

permeabilized with 70% ethanol, then incubated with PI in 1XPBS (phosphate buffered saline). The number of migratory cells per membrane was counted with an Olympus upright fluorescence microscope with a 40x objective. Non-specific migration as measured on chambers with no matrigel or laminin was subtracted.

Toxin B Inhibition

Clostridium difficile Toxin B was purchased from Calbiochem (CA). Cells were treated with 2 ng/ml Toxin B for 24 hours before being subjected to haptotaxis assay.

Guanine Nucleotide Binding

Cell lysates were incubated for 15 min at 30 °C in the presence of 10 mM EDTA and 100 μM GTPγS or 1 mM GDP to facilitate nucleotide exchange as described in (Knaus et al., 1992). The loading reaction was stopped by addition of 60 mM $MgCl_2$.

Rac, Cdc42, and Rho Activity Assays

Rac and Cdc42 activity assays were performed as described in (Benard et al., 1999), Rho activity assays were performed as described in (Ren and Schwartz, 2000), with minor modifications. Briefly, cells were washed twice with 1X PBS, lysed with 1X ice cold lysis buffer, and scraped from the plate. Lysates were then incubated at 4° for 1 hour with 10 μg of PAK-PBD Protein GST Beads (Cytoskeleton Inc., CO) for Rac and Cdc42, or Rhotekin-RBD Protein GST Beads (Cytoskeleton Inc., CO) for Rho activity. The bead pellet was then washed once with wash buffer containing 1% Nonidet P-40

(Calbiochem, CA) and twice without Nonidet P-40. The bead pellet was finally suspended in 20 µl Laemelli sample buffer. Proteins from total cell lysate, as well as the bead pellet, were separated by 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and blotted for the appropriate GTPase using a monoclonal anti-Rac (clone 32A8) antibody (Upstate Biotechnology, NY), a rabbit polyclonal anti-Rho -A, -B, -C antibody (Upstate Biotechnology, NY), a mouse monoclonal anti-Cdc42 (clone 44) antibody (Transduction Laboratories, CA), or a goat polyclonal anti-RhoC antibody (Santa Cruz Biotechnology, CA). Immunoblots were detected with the SuperSignal West Femto-Substrate chemiluminescence kit (Pierce Endogen, IL) and Kodak Biomax MR film (Fisher Scientific, TX).

3.3 Results

α6 Integrin expression among the panel of metastatic variants.

The isolated MDA-MB-435 cell variants were found to express differential levels of α6 integrin, as measured by flow cytometry (Mukhopadhyay et al., 1999). To confirm this result, total endogenous protein expression of α6 integrin was measured by western blot analysis. Increasing levels of α6 integrin were found to correlate with increasing metastasis in the four isolated variants of MDA-MB-435 variants (Figure 3.1).

Motile morphology of MDA-MB-435 metastatic variants correlates with metastatic efficiency.

To understand the role of the Rho GTPases in metastatic breast cancer, we used isolated variants of the MDA-MB-435 metastatic breast cancer cell line that had been cycled through the nude mouse model of experimental metastasis to determine metastatic efficiency (Mukhopadhyay et al., 1999). The results identified MDA-MB-435α6HG6 as the variant most likely to produce distant metastasis, followed by the parental MDA-MB-435, then MDA-MB-435α6LF9, and finally MDA-MB-435Br1 (Mukhopadhyay et al., 1999).

An invasive cellular phenotype can be indicative of metastatic behavior (Schmitz et al., 2000). Rac-induced membrane ruffles, or lamellipodia, have been shown not only to be important structures in cellular motility, but have also been shown to play a key role in invasion with respect to metastatic progression (Ridley, 2001; Condeelis et al., 2001). Rac-induced lamellipodia contain cell-substratum contacts, or focal adhesions, and aberrant focal adhesion expression has also been associated with malignant progression (Schlaepfer et al., 2004). Therefore, we investigated the correlation between cytoskeletal phenotype, focal adhesion contacts, and metastatic efficiency. Our data shows a direct correlation between increased lamellipodia expression and increased metastatic efficiency (Figure 3.2). The most metastatic variant, MDA-MB-435α6HG6, exhibits a strikingly different phenotype than the other variants, including an increased number of focal adhesions as well as a cross-linked actin network. In fact, increasing focal adhesion expression also correlates with increasing metastatic efficiency (Figure 3.3a). However,

individual MDA-MB-435α6HG6 (most metastatic) cells were 1.5 times larger than other variants (data not shown). Thus, the data was compiled as focal adhesions per cell area (Figure 3.3b). This correlation between lamellipodia, focal adhesions, and metastatic potential strongly suggests an increase in Rac activity among those variants with increased metastatic efficiency.

Subcellular distribution of focal adhesions and filamentous actin subsequent to cellular polarization during the wound healing response.

Actively motile cells polarize to form leading edge lamellipodia toward the direction of migration, which is one of the initial steps of intravasation (Condeelis et al., 2001). To induce cellular polarization, the four cell variants were stimulated by wounding a confluent monolayer to produce a motile response. The cells were then immunostained with rhodamine phalloidin to visualize f-actin structures and an anti-phosphotyrosine to visualize focal adhesions. Focal adhesions can be classified into two distinct categories: smaller, more compact focal complexes and longer, stress-fiber associated focal contacts (Zamir and Geiger, 2001). Focal complexes, induced by the activation of the small GTPase Rac, are found at the leading edge of lamellipodia and are responsible for the generation of strong propulsive forces in migrating fibroblasts (Nobes and Hall, 1995; Clark et al., 1998; Rottner et al., 1999; Beningo et al., 2001; Clark et al., 1998; Rottner et al., 1999; Beningo et al., 2001). Subsequent to their formation, focal complexes will develop into focal contacts as a consequence of the activation of Rho (Clark et al., 1998; Rottner et al., 1999).

In this study, we find that cellular polarization in the more metastatic variants tends to elicit wider membrane ruffles as well as more cell area invasion into the wound space (Figure 3.4, upper panels). Upon stimulation by wounding, the most metastatic MDA-MB-435α6HG6 variant demonstrates the most invasive phenotype, exhibiting marked lamellipodial invasion into the wound space (Figure 3.4, upper left panel). The parental cell line, MDA-MB-435, also highly metastatic, exhibits a comparatively invasive phenotype to the MDA-MB-435α6HG6, but with smaller lamellipodia and is consequently less invasive into the wound space (Fig 3.4, upper right). The two remaining variants, MDA-MB-435α6LF9 and MDA-MB-435Br1, exhibit similar phenotypes to each other in response to wounding (Figure 3.4, lower panels). The cell-surface F-actin containing structures of these cell variants extending into the wound space are more elongated and slender than the wider lamellipodia of the more metastatic strains. In addition, the points of contact between ECM and cell surface of these less metastatic variants appear to be mature focal contacts, indicating a more stationary cell and a less motile phenotype (Figure 3.4). The same points of contact in the more metastatic variants appear to be more like nascent focal adhesions, indicating a more motile and invasive phenotype (Figure 3.4).

Migratory phenotype of MDA-MB-435 variants correlates with metastatic efficiency.

Subsequent to the epithelial to mesenchymal transition, cells must first migrate away from the primary tumor through the basal lamina to begin the process of

establishing sites of secondary tumorigenesis. Therefore, increased cell migration in malignant cells is thought to be closely linked to invasion and metastasis (Ridley et al., 2003). Upon investigation into migratory behavior of the cell variants, we found a correlation between increased metastatic potential and increased migration (Figure 3.5a). Because the Rho family of small GTPases, namely Rac, Rho, and Cdc42, are essential to cell motility, we used *Clostridium difficile* toxin B to inhibit the Rho family in these cell variants. Subsequent to treatment with toxin B, the most metastatic variant MDA-MB-435α6HG6 exhibited a 2-fold decrease in migration to basal lamina, while the others exhibited a substantial, but not significant, decrease in migration (Fig 3.5b).

Increased Rac and Rho activity directly correlate with metastatic potential.

Increases in activity levels of the Rho proteins Rho, Rac, and Cdc42 have been shown to be accountable for the promotion of tumor cell invasiveness (Fritz et al., 2002; Price and Collard, 2001). Therefore, we investigated the activity levels of these proteins in all MDA-MB-435 metastatic variants. To determine the relative amounts of activated Rho in the variant panel, the RBD-GST activity assay was used (Ren and Schwartz, 2000). Total endogenous Rho protein expression varied among the variants, with the more metastatic variants expressing more endogenous Rho protein than the less metastatic variants. However, increased Rho protein activity was found to directly correlate with increased metastatic potential (Figure 3.6a). Loading cell lysates with a non-hydrolyzable GTP analog, GTPγS, showed a differential binding ability of the Rho proteins among the four variants. This result could be due to differential endogenous

protein expression of the different Rho isoforms, RhoA and RhoC. In fact, endogenous expression of RhoA was equal among the variants, but endogenous RhoC expression was greatly increased in the most metastatic variant (Figure 3.6b).

To determine the relative amounts of activated Rac and Cdc42 in the variant panel, we used the PBD-GST activity assay (Benard et al., 1999). While total endogenous Rac protein expression remains equal among the cell variants, Rac protein activity directly correlates with increased metastatic potential (Figure 3.7a). Loading cell lysates with a non-hydrolyzable GTP analog, GTPγS, showed a relatively equal GTP-binding ability of the Rac protein among the four variants. Therefore, all Rac expressed in the variants of the metastatic panel can be activated to the same extent. Thus, endogenous activators of Rac appear to have increased activity in the more metastatic cell variants. Endogenous Cdc42 protein expression differed among the variants: the more metastatic variants expressed higher levels of endogenous Cdc42 than the less metastatic variants (Figure 3.7b). However, no active Cdc42 protein could be detected. Again, GTPγS loading showed the ability of the Cdc42 proteins to bind GTP and become active.

Blocking Cdc42 activation has no significant effect on cell migration.

Hyperactive Cdc42 has been implicated in tumor cell invasion due to its effects on the actin cytoskeleton (Bouzahzah et al., 2001). Additionally, EGFR overexpression has been shown to be responsible for this hyperactivation (Sturge et al., 2002). To determine a role for Cdc42 in the migration of highly metastatic cells, we expressed vector alone and a dominant negative myc-Cdc42(T17N) construct in the highly

metastatic MDA-MB-435α6HG6 cell variant and subjected both to a migration assay. We found that Cdc42(T17N) did not significantly inhibit migration as compared to the vector alone control (Figure 3.8a). However, when we expressed vector alone, dominant negative Rac1(T17N) or dominant negative Rac3(T17N), we found a significant inhibition (p value>0.01) of migration as compared to the vector control (Figure 3.8b). Therefore, Rac activity appears to be essential for the migration of highly metastatic cells, while Cdc42 does not.

3.4 Discussion

The present study illustrates a correlation between the activated Rho proteins Rac and Rho, the invasive phenotype, and the increased metastatic capability of the human breast cancer cell variants of the MDA-MB-435 cell line. Rho proteins have been both directly and indirectly associated with the transformation from primary tumor cells to highly motile and invasive malignant cells (Kleer et al., 2002; Silva et al., 2000; Bourguignon et al., 2000; Bouzahzah et al., 2001). Invasive phenotypes, including aberrant focal adhesions and increased numbers of lamellipodia, have also been associated with metastatic progression and increased cellular motility (Kassis et al., 2001; Ridley, 2001; Sahai and Marshall, 2002).

Migratory Phenotype and Metastatic Progression

It has been shown that the intravasation of cancer cells begins with directed lamellipod extension (Condeelis et al., 2001). We confirm this finding by demonstrating lamellipod extension directly into the wound space by the more metastatic MDA-MB-435 variants. In addition, we demonstrate that the overall size of the lamellipod, as well as the quantity of overall F-actin staining, directly correlates with increasing metastasis. Focal complexes have been shown to be associated with both lamellipodia and the generation of strong propulsive forces in migrating fibroblasts (Nobes and Hall, 1995; Clark et al., 1998; Rottner et al., 1999; Beningo et al., 2001). Again, we validate these observations by demonstrating that in cells growing in serum, increasing focal adhesion number per cell area directly correlates with increasing metastatic potential. This finding suggests that cells exhibiting larger lamellipodia will migrate and invade in greater numbers than those with the more slender uropodia containing less focal complexes. Indeed, we find this to be the case across reconstituted basement membrane. As predicted, the more metastatic cells, or those presenting larger lamellipodia and more focal complexes, invaded and migrated through reconstituted basal lamina faster than those forming slender uropodia upon stimulation. In conclusion, the more metastatic cells tend to exhibit clear morphological as well as physiological differences from the less metastatic cells.

Rac and Cdc42 Activation and Metastatic Progression

Increased activation of the small GTPases Rho, Rac, and Cdc42 have been strongly implicated in malignant progression (Schmitz et al., 2000; Evers et al., 2000; Jaffe and Hall, 2002; Steeg, 2003). Several studies have shown that increased Rac1 or RhoA,C signaling via increased protein activation can promote the acquisition of an invasive phenotype (Price and Collard, 2001; Bourguignon et al., 2000; Otsuki et al., 2001; Zhuge and Xu, 2001). In the present study, we corroborate these findings by demonstrating that metastatic potential directly correlates with levels of Rac activation. In addition, we substantiate the idea that increased Rac activity correlates with increased focal complex and lamellipodia formation, as well as increased migration across basal lamina. However, we could not find the same correlation with the small GTPase Cdc42. The disregulation of Cdc42 has been implicated in tumor cell invasion due to its effects on the actin cytoskeleton, via its downstream effector WASP, which activates actin nucleation by stimulating Arp2/3 (Sturge et al., 2002). Several studies have implicated Cdc42 in regulating the initial cell polarization necessary for directed motility (Srinivasan et al., 2003; Wedlich-Soldner et al., 2003). In the present study, we found that levels of activated Cdc42 were so low as not to be detected by our techniques. Although in opposition to other findings, this data does not support a direct Cdc42-mediated role for cell polarization during the migration of breast cancer cells. Moreover, Cdc42 is known to activate Rac, so it is possible that we were detecting a temporal effect of Cdc42 on Rac activity.

Increased α6 Integrin Expression and Rho GTPase Activation is Linked to Increased Metastatic Potential

Because these variants were sorted according to α6 integrin expression, and increased α6 integrin expression correlates with metastatic capability, it is possible that aberrant α6 integrin expression is responsible for the variations in metastatic capability of these cells (Mukhopadhyay et al., 1999; Shimizu et al., 2002; Friedrichs et al., 1995; Tagliabue et al., 1998). In MDA-MB-435 cell lines, β1 integrin dimerizes with α6 integrin to form the transmembrane heterodimer that binds laminin (Wewer et al., 1997). Significantly, both the overexpression and stimulation of β1 integrin have been found to increase Rac activity and lamellipodia formation (Sturge et al., 2002; Miao et al., 2002). Moreover, it has been shown that integrin clustering and subsequent Rac activation can lead to invasion via the GEF Vav2 (Cho and Klemke, 2000). It has also been demonstrated that Vav2 is a crucial downstream component in EGFR- and PI 3-kinase-dependent Rac activation upon integrin-mediated cell adhesion (Marcoux and Vuori, 2003). Therefore, it is possible that increased a6b1 integrin expression in out panel of metastatic variants is causing the upregulation of Rac activation via the GEF Vav2. However, we present no direct evidence for this activation, and thus required further experimentation.

It is possible to block the activation α6 integrin, and subsequently α6β1 integrin signaling, with the α6 integrin-blocking monoclonal antibody GoH3 (Jiang et al., 2001; Dangerfield et al., 2005). To show that blocking α6β1 adhesion and subsequent signaling is responsible for the Rho GTPase activation, the cell variant with the highest endogenous

Rac activation should be used (MDA-MB-435α6HG6). Rac and Rho activity assays should be performed subsequent to α6 integrin blocking. If α6β1 integrin is responsible for Rho protein activation, a decrease in Rho and Rac activity should be seen. Furthermore, GEF activation assays should also be performed to determine the activation pathway linking α6β1 integrin engagement to Rho protein activation.

Rho Activation in Metastatic Progression

The role of the Rho protein in cancer cell invasion is somewhat controversial. Some studies find that overexpression of Rho has little effect, while others have demonstrated a positive role for Rho in tumor cell migration and invasion (Stam et al., 1998; Itoh et al., 1999; O'Connor et al, 2000). The reason for this inconsistency is based on the fact that active Rho performs two roles regarding migration: Rho promotes stress fiber formation while at the same time facilitates cell body contraction (Ridley, 2001). Therefore, cellular effects caused by the disregulation of Rho is dependent on cell type, and tends to reflect the basal levels of stress fibers and focal adhesions found within the cell (Cox and Huttenlocher, 1998; Ridley, 2001). RhoC, a Rho isoform associated primarily with the contractility of the actin cytoskeleton, has recently been identified as an oncogene in breast cancer that can promote the metastatic phenotype (van Golen et al., 1999). Although total protein expression of Rho as detected with an antibody to the -A, -B, and –C, isoforms demonstrates equal expression across all cell strains, western blotting with a RhoC-specific antibody revealed increased endogenous RhoC expression in the more metastatic variants, while blotting with a RhoA-specific antibody detected

little difference among the variants. Activity assays detected increased overall Rho activity in the more metastatic variants, but this variation could possibly be due to the activity of RhoC, and not Rho -A or -B. Due to the evidence that the increased activation of Rac and Rho correlates with increased invasion and metastasis, we substantiate the idea that migratory behavior, and subsequent tumor cell invasion, is a result of a reciprocal balance between Rac and Rho activities (Evers et al., 2000).

Conclusion

This study, for the first time, demonstrates a direct correlation between increased Rac and Rho activity and increased metastatic potential. Moreover, for the first time, this study suggests a correlation between the increased expression of α6β1 integrin, increased Rac activity, and increased RhoC expression. It is clear from the results that all of these factors increase migratory and adhesive properties *in vitro*. However, whether the correlation is direct or indirect remains yet to be determined.

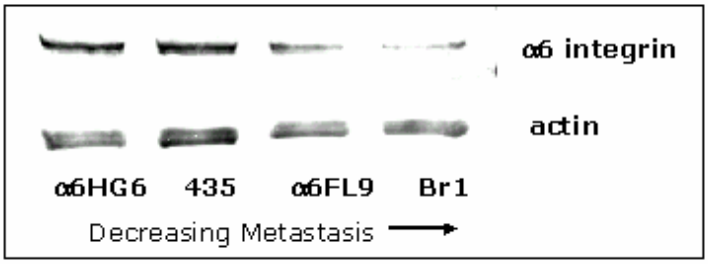


Figure 3.1. α6 Integrin expression in MDA-MB-435 metastatic variant panel. Whole cell lysates of MDA-MB-435α6HG6, MDA-MB-435, MDA-MB-435α6LF9, and MDA-MB-435Br1 were subjected to SDS-PAGE followed by western blot analysis for integrin α6. Equal loading of lanes was maintained by lysing equal numbers of cells per variant, followed by a total protein assay, and shown by western blot analysis for F-actin.

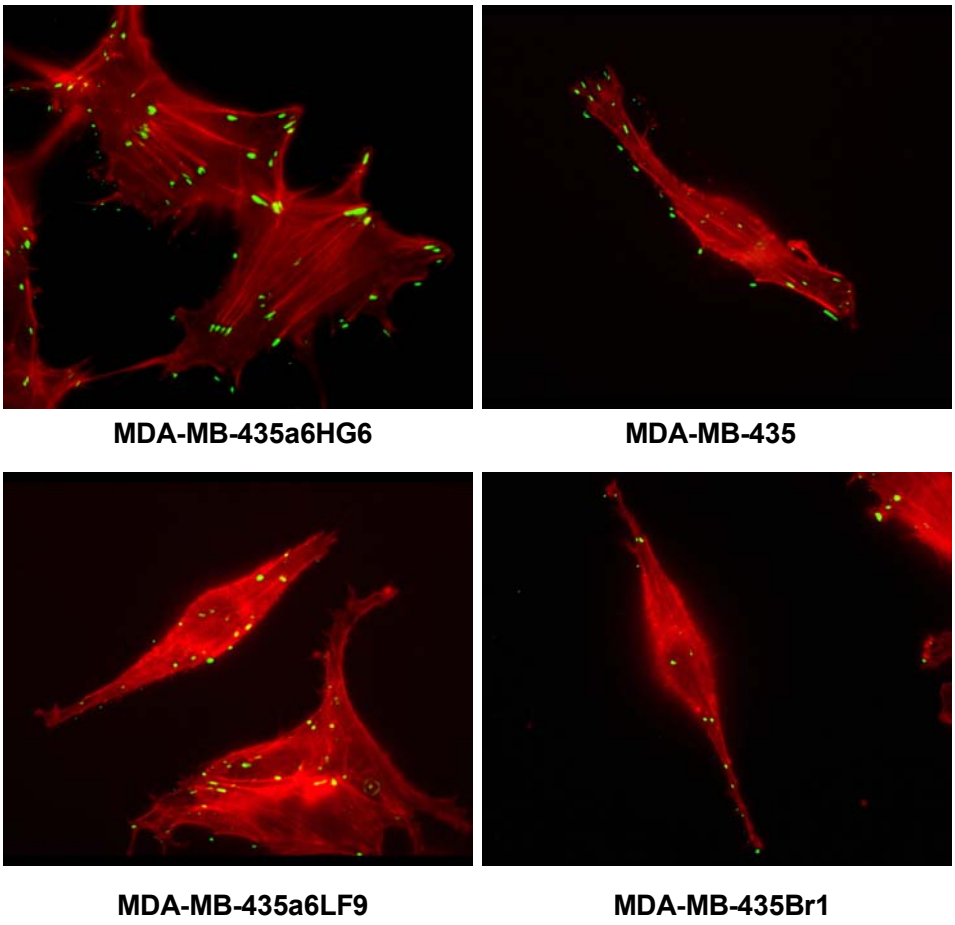


Figure 3.2. Characterization of cytoskeletal structures and focal adhesion distribution in MDA-MB-435 metastatic variants. Each of the MDA-MB-435 metastatic variants were plated onto glass coverslips. Actin was then visualized with rhodamine phalloidin and focal adhesions were visualized with an anti-p-tyro antibody followed by an FITC conjugate.

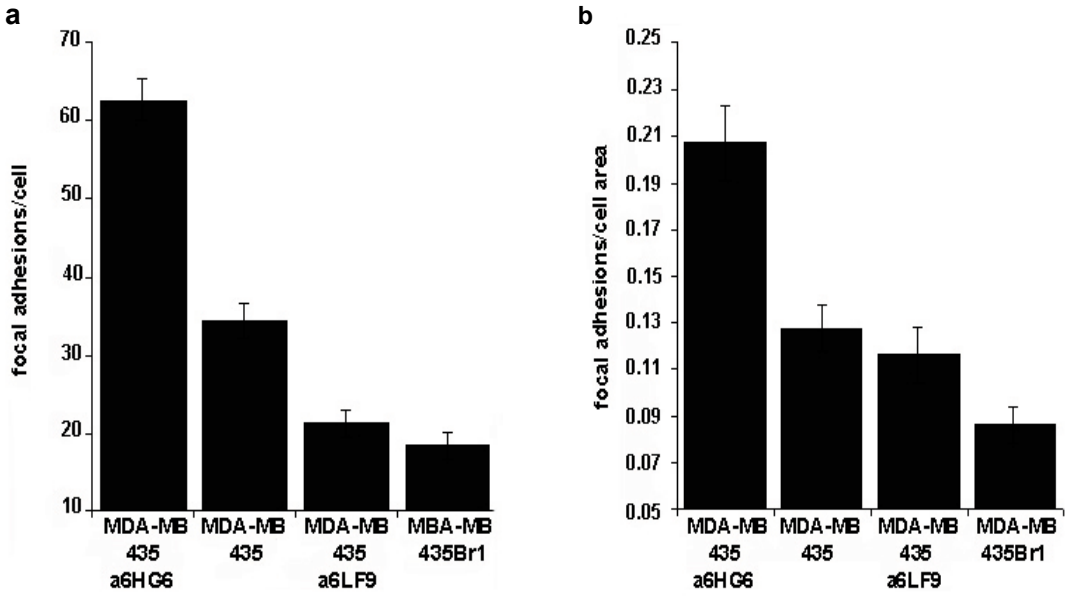


Figure 3.3. Quantitation of focal adhesion distribution in MDA-MB-435 metastatic variants. (a) Focal adhesions were counted on a total of 100 individual cells per variant. Data shown are the average of 50 cells per variant, with the bars representing standard error of the mean, and are representative of three independent experiments. **(b)** Cell area was measured on 50 individual cells per variant using Spot Digital Camera Software. Focal adhesion number was divided by cell area and plotted on the y-axis. Bars represent (+/-) SEM, and are representative of three independent experiments.

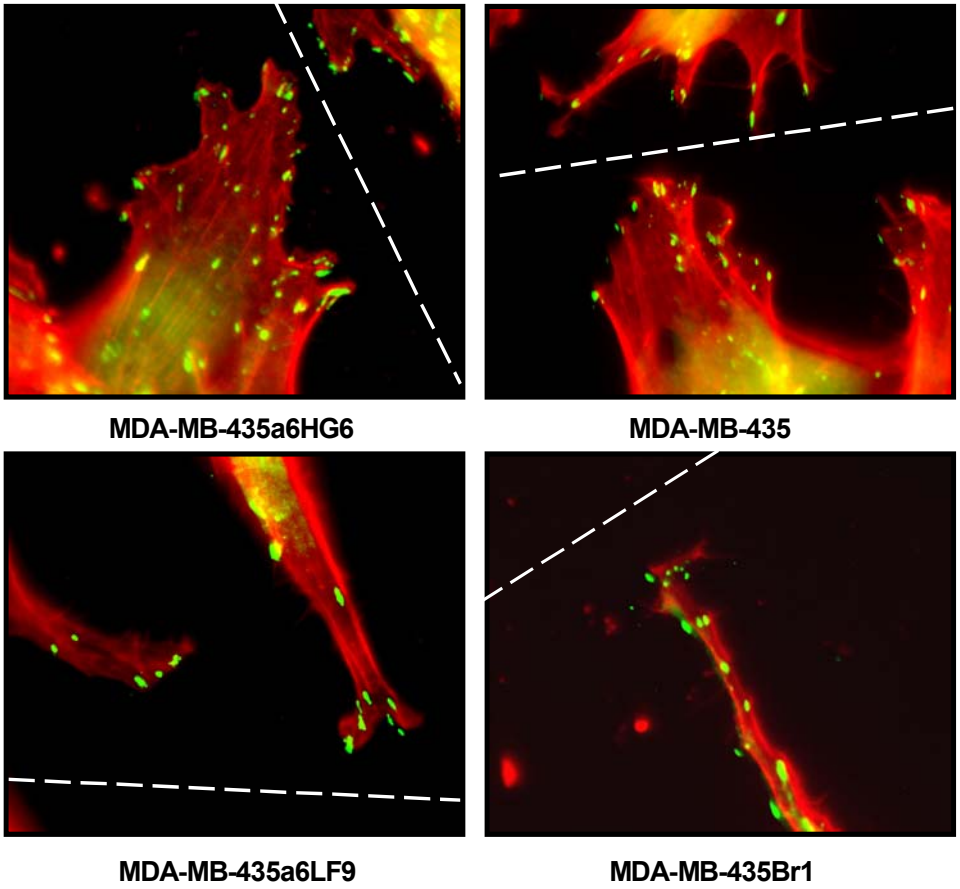


Figure 3.4. Migratory morphology of MDA-MB-435 variants. Cells were grown to confluent monolayers then wounded to stimulate the motile response. Dotted white line represents the wounding site. Actin was then visualized with rhodamine phalloidin and focal adhesions were visualized with an anti-phosphotyrosine antibody followed by an FITC conjugate.

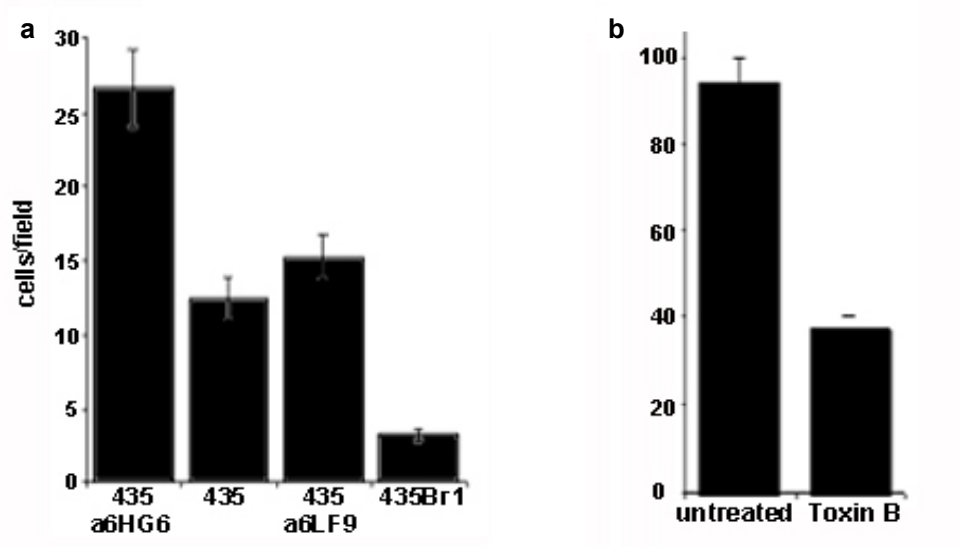


Figure 3.5. Haptotaxis assays of MDA-MB-435 metastatic variants. (a) Each variant was adjusted to 500,000 cells and applied to Transwell chambers in a basement membrane haptotaxis assay. Cells migrating to the underside of the chamber were stained with PI and counted under (400X). Bars represent +/- SEM. Data is representative of three independent experiments. **(b)** Cells either treated with Toxin B (Toxin B) or untreated (untreated) were subjected to a haptotaxis assay. Each group was adjusted to equal concentrations and applied to Transwell chambers. Cells migrating to the underside of the chamber were stained with PI and counted under (400X). Bars represent +/- SEM.

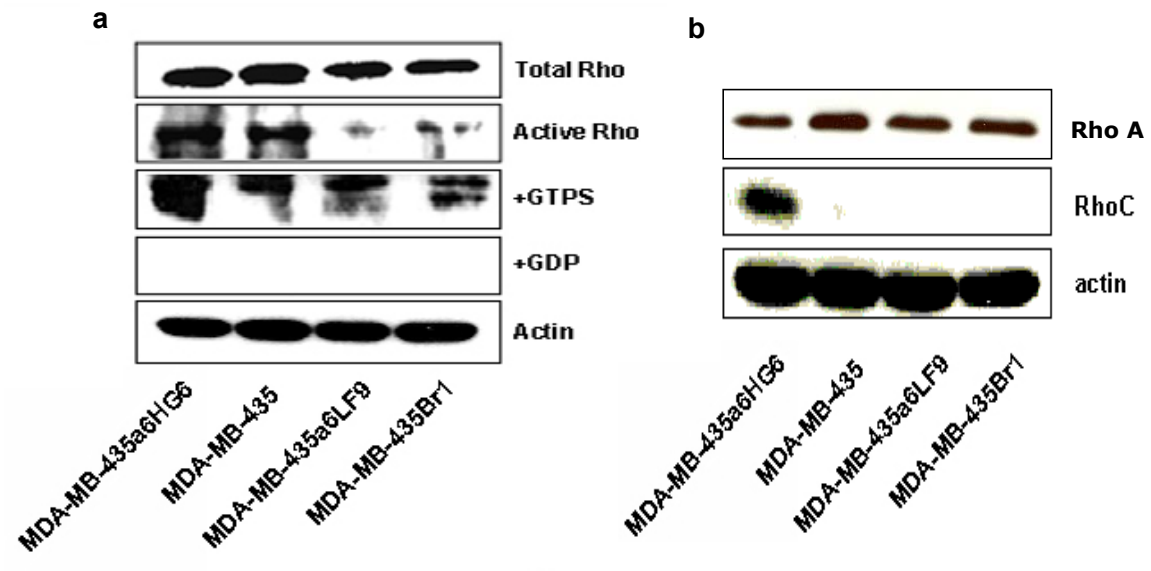


Figure 3.6. Rho expression and activity in MDA-MB-435 metastatic variants. Whole cell lysates of all variants were subjected to SDS-PAGE followed by western blot analysis for total Rho expression using an anti-Rho (A,B,C) antibody, an anti-RhoA specific or an anti-RhoC specific antibody. Rho activity was assayed using the GST-RBD activity assay. A non-hydrolyzable GTP analog, GTPγS, was used as the positive control; GDP alone was used for the negative control. Equal loading of lanes was maintained by performing a total protein assay and is confirmed by western blot analysis for total actin. Results are representative of three to five independent experiments.

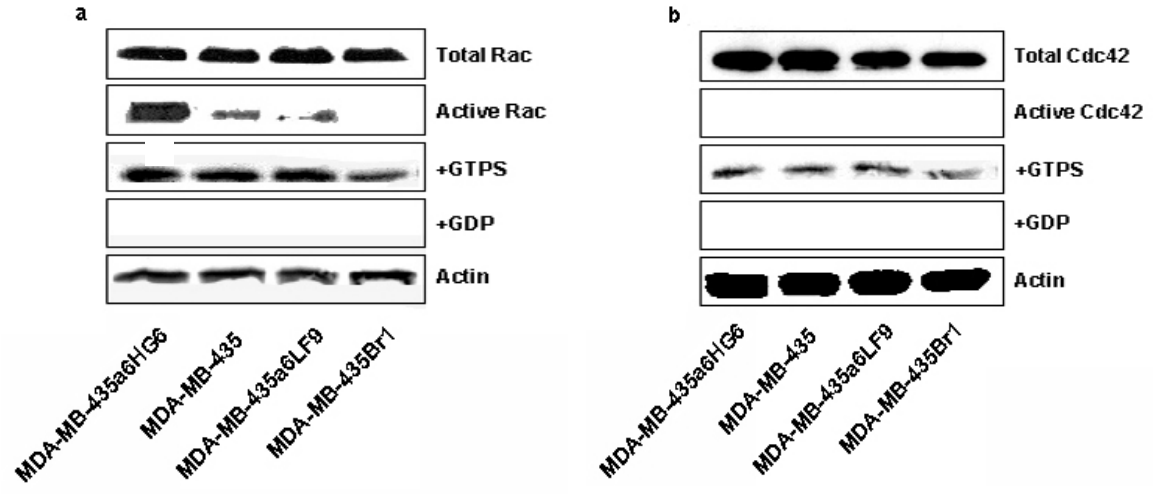


Figure 3.7. Rac and Cdc42 activity in MDA-MB-435 metastatic variants. Whole cell lysates of all variants were subjected to SDS-PAGE followed by western blot analysis for total Rac **(a)** using an anti-Rac antibody and total Cdc42 **(b)** using an anti-Cdc42 antibody. Rac and Cdc42 activity were assayed using the PAK-PBD activity assay. A non-hydrolyzable GTP analog, GTPγS, was used as the positive control; GDP alone was used for the negative control. Equal loading of lanes was maintained by performing a total protein assay and is confirmed by western blot analysis for total actin. Results are representative of three to five independent
experiment

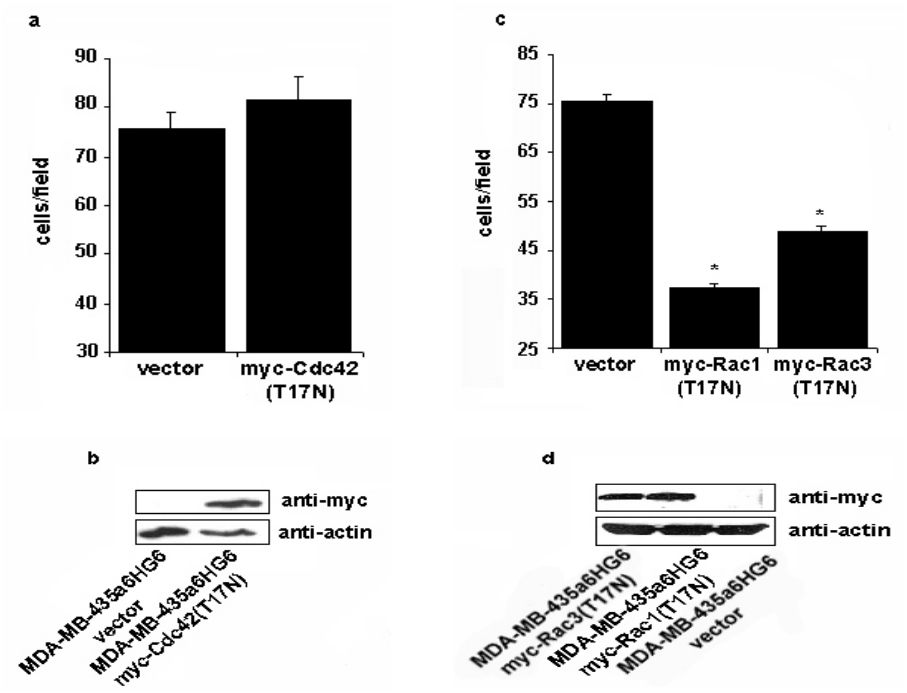


Figure 3.8. Migration of MDA-MB-435α6HG6 cells expressing dominant negative Rac and Cdc42 mutants. (a) MDA-MB-435α6HG6 cells transiently expressing vector alone or myc-Cdc42(T17N) were subjected to a haptotaxis assay. Cells migrating to the underside of the membrane were stained with PI and counted under (400X). Bars represent (+/-) SEM. Equal loading was confirmed by a total actin blot, myc-Cdc42(T17N) expression confirmed by western blot with anti-myc. **(b)** MDA-MB-435α6HG6 cells transiently expressing vector alone, myc-Rac1(T17N), or myc-Rac3(T17N) were subjected to a haptotaxis assay. Bars represent (+/-) SEM, equal loading was confirmed by total actin blot. Myc-Rac1(T17N) and myc-Rac3(T17N) expression were confirmed by both anti-Rac and anti-myc. An asterix indicates a statistically significant difference compared to the control, vector alone, as determined by a Student's *t*-test ($P<0.05$).

4. Rac1 and Rac3 Activation is Involved in the Invasive and Metastatic Phenotype of Human Breast Cancer Cells

4.1 Introduction

Cancer metastasis is a multi-faceted process requiring the disregulation of numerous signaling pathways, including those associated with cell adhesion and motility. The initial steps of metastasis require the acquisition of a motile phenotype in order to traverse tissue boundaries, while the later stages require the activation of cell adhesion to facilitate the extravasation of malignant cells (Sahai and Marshall, 2002). Activation of the Rho family GTPases Rac and Cdc42 is a critical event in the integrin and growth factor-mediated regulation of cellular migration and adhesion, which implicates the hyperactivation of these proteins in the progression of metastatic disease (Miranti and Brugge, 2002).

Rac and Cdc42 in Breast Cancer Metastasis

The activation of Rac and Cdc42 is critical for initiating cell motility and adhesion via the dynamic turnover of cell-substratum contacts (focal adhesions) and the nucleation of actin monomers leading to the assembly of actin filaments necessary for cell movement (Hynes, 2002). Activation of the appropriate levels of these proteins, together with temporal and spatial coordination, must be precisely regulated in order to achieve normal cellular function (Price and Collard, 2001). Aberrant Rac and Cdc42

activity have been recently associated with invasive and malignant behavior in a variety of cell types, including hepatocarcinoma, breast carcinoma, and melanoma (Lee et al., 2004; Bouzahzah et al., 2001; Uhlenbrock et al., 2004). However, breast tissue sample analysis has shown that the contribution of the Rac and Cdc42 proteins to tumor cell invasion in breast cancer is not due to genetic mutation, but is due instead to changes in the activity levels of these proteins caused by hyperactivation of upstream activators (Fritz et al., 2002; Price and Collard, 2001). Yet, a direct correlation between Rac and Cdc42 protein activity states and metastatic progression in human breast cancer remains to be demonstrated.

The Rac-like Subfamily of Rho GTPases

The Rac-like subfamily of Rho GTPases includes Rac1, the myeloid-lineage specific Rac2, and the subsequently cloned Rac3 protein (Haataja et al., 1997). Because Rac2 is found only in myeloid-lineage cells, only Rac1 and Rac3 are thought to be involved in breast cancer metastasis. Exhibiting a 92% identity to Rac1, Rac3 differs from Rac1 in the C-terminus, a region essential for subcellular localization, and in the insert region, a region necessary for regulatory protein binding (Haataja et al., 1997; Chou and Blenis, 1996). In fact, some differences have been found between Rac1 and Rac3 function. For example, Rac3 has been found to be more highly expressed in neural tissue than is Rac1 (Bolis et al., 2003). This differential distribution is thought to support a role for Rac3 specifically in the remodeling of Purkinje cell neuritic terminals at the time of synaptogenesis (Bolis et al., 2003). Rac3 has been found to interact with the

integrin-binding protein calcium and integrin-binding (CIB) protein, a protein with which neither Rac1 nor Rac2 interact (Haataja et al., 2002). This differential binding is thought to implicate Rac3 specifically in integrin-associated cytoskeletal reorganization during αIIBβ3-mediated adhesion (Haataja et al., 2002). Furthermore, Rac3, but not Rac1, was found to control proliferation in breast cancer cells (Mira et al., 2000).
However, a direct role for Rac3 in breast cancer invasion and metastasis has never been substantiated.

To further understand the molecular mechanisms of the small GTPases Rac and Cdc42 in human breast cancer, we used a panel of metastatic variants derived from the parental MDA-MB-435 breast cancer cell line (Mukhopadhyay et al., 1999). Within this panel, we found a direct correlation between both the invasive phenotype and enhanced migratory ability and increased metastatic potential (Chapter 3). Moreover, we found that increased Rac, but not Cdc42, activation correlated with increased metastatic potential (Chapter 3).

Previously, Rac1 was shown to play a critical role in rat mammary tumor cell growth and metastasis *in vivo* (Bouzahzah et al., 2001). To establish a role for both Rac1 and Rac3 in human breast cancer, we carried out a comparative study between the two isoforms. Dominant active Rac1 or Rac3 mutants were expressed in the least metastatic cell variant of our panel, while dominant negative Rac1 or Rac3 mutants were expressed in the most metastatic cell variant. Dominant active Rac expression of either isoform resulted in an aggressive phenotype, as well as significant increases in adhesion, migration, and invasion. Conversely, dominant negative expression of either isoform

resulted in significant decreases in adhesion, migration, and invasion. Moreover, low metastatic cell lines stably expressing dominant active Rac1 or Rac3 proteins caused metastatic lesions in the lung of the nude mouse, as compared to the control. Highly metastatic cell lines stably expressing dominant negative Rac 1 or Rac3 blocked metastasis to the lung of the nude mouse. Taken together, these data suggest a direct role for both Rac1 and Rac3 proteins in the metastatic progression of human breast cancer.

4.2 Materials and Methods

Cell Culture

The human breast cancer cell lines variants MDA-MB-435α6HG6 and MDA-MB-435Br1 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco™, CA) with 10% fetal bovine serum (FBS) (Tissue Culture Biologicals, CA) and cultured in a humidified 5% CO_2 atmosphere at 37°C.

DNA Constructs, Transfections, and Stable Cell Selection

Rac1 mutant cDNA (Myc-Rac1(G12V) and Myc-Rac1(T17N)) were generous gifts from Dr. Gary Bokoch of the Scripps Research Institute (La Jolla, CA). Rac3 mutant cDNA (Myc-Rac3(G12V) and Myc-Rac3(T17N)) were generous gifts from Dr. Ulla Knaus of the Scripps Institute (La Jolla, CA). Mutant Rac cDNAs were digested out of the pRK5myc vector and inserted into the multiple cloning site of the pIRESneo2 vector (Clontech).

pIRESneo2 vector alone, or vectors encoding Myc-Rac1(G12V), Myc-Rac1(T17N), Myc-Rac3(G12V), or Myc-Rac3(T17N) were transfected into cell variants using Lipofectamine Plus Reagent (Gibco^{TM}, CA). Maximal expression was achieved 24-48 hours post transfection.

Cells expressing constructs were selected in 1 mg/ml G418 Sulfate (Fisher Scientific, TX) for 3 weeks. Subsequent to selection, colonies were picked and subcloned in 1mg/ml G418 Sulfate for an additional 3 weeks.

Rac Activity Assay

Rac activity assays were performed as described in (Benard et al., 1999), with minor modifications. Briefly, cells were washed twice with 1X PBS, lysed with 1X ice cold lysis buffer, and scraped from the plate. Lysates were then incubated at 4° for 1 hour with 10 μg of PAK-PBD Protein GST Beads (Cytoskeleton Inc., CO) for Rac activity. The bead pellet was then washed once with wash buffer containing 1% Nonidet P-40 (Calbiochem, CA) and twice without Nonidet P-40. The bead pellet was finally suspended in 20 μl Laemelli sample buffer. Proteins from total cell lysate, as well as the bead pellet, were separated by 12% SDS-PAGE gel, transferred to a nitrocellulose membrane, and blotted for the appropriate GTPase using a monoclonal anti-Rac (clone 32A8) antibody (Upstate Biotechnology, NY) or an rabbit polyclonal anti-actin antibody (Sigma, MO). Immunoblots were detected with the SuperSignal West Femto-Substrate chemiluminescence kit (Pierce Endogen, IL) and Kodak Biomax MR film (Fisher Scientific, TX).

Immunofluorescence Microscopy

Cells in culture were placed on glass coverslips, fixed in 3.7% formaldehyde (Sigma Chemical Corp., MO), permeabilized with 0.5% Triton X-100 (Sigma, MO), and blocked with 5% goat serum (Gibco^{TM}, CA) and 5% bovine serum albumin (BSA) (Sigma Chemical Corp., MO). Cells were then stained with rhodamine phalloidin (Molecular Probes, OR) to visualize F-actin, and a mouse monoclonal anti-phosphorylated tyrosine antibody, clone 4G10 (Upstate Biotechnology, NY), followed by FITC-conjugated goat anti mouse IgG (ICN Biomedicals Inc., CA) to visualize focal adhesions. Cells were imaged with either an Olympus upright fluorescence microscope or an inverted confocal microscope with fluorescence and DIC capabilities. Images were overlayed with Spot Advanced digital camera software (Diagnostic Instruments Inc., MI).

Adhesion Assays

Cell adhesion assays were performed according to (Klemke et al., 1998). Briefly, glass coverslips (Fisher Scientific, TX) were coated with laminin (Gibco BRL, MD). Proteins were allowed to bind over night at 4° before the coverslips were blocked for 1 hour with 1% heat-denatured bovine serum albumin (BSA) (Sigma Chemical Corporation, MO) in 1X PBS. Cells (10^5) were added to the wells and allowed to adhere for 15 minutes. Non-adherent cells were removed, and the adherent cells were fixed in 3.7% formaldehyde (Sigma Chemical Corp., MO). The number of cells per coverslip was counted with an Olympus upright microscope with a 40x phase contrast objective.

Nonspecific cell adhesion as measured on poly-L-lysine coated coverslips has been subtracted.

Haptotaxis Migration and Invasion assays

Cell migration and invasion assays were performed as described in (Klemke et al., 1998). Briefly, modified Boyden chambers (tissue culture treated, 6.5 mm diameter, 10μm thickness, 8 μm pores, Transwell®, Costar Corp., Cambridge, MA) containing polycarbonate membranes were coated with matrigel (Fisher Scientific, TX) or laminin (Gibco BRL, MD) on the underside of the membrane (migration), or the upperside of the membrane (invasion). For invasion assays, cells chemotracted to media supplemented with 10% fetal bovine serum (FBS) (Tissue Culture Biologicals, CA). Serum starved cells (10^6 cells) were added to the upper surface of each migration chamber and allowed to migrate to the underside of the membrane for 4 hours (migration) or 24 hours (invasion). The non-migratory cells on the upper membrane surface were removed, and the migratory cells attached to the bottom surface of the membrane were stained with propidium iodide (CalBioChem-Novabiochem Corp., CA). For PI staining, cells were fixed and permeablized in 70% ethanol and then incubated with 40 μg/mL PI in 1X PBS. The number of migratory cells per membrane was counted with an Olympus upright fluorescence microscope with a 40x objective for migration assays and a10x objective for invasion assays. Non-specific migration as measured on chambers with no chemotractant has been subtracted.

Flow Cytometry

Stable cell lines were harvested from culture with trypsin, fixed and permeablized with 70% ethanol, and stained with PI for cell cycle analysis. Analysis was performed on a Coulter Epics Elite Flow Cytometer (Miami, FL) and analyzed by MultiCyle DNA analysis software (Phoenix Flow Systems, San Diego, CA).

Nude Mouse Model of Experimental Metastasis

Female athymic nude mice were purchased from Charles River Laboratories (Wilmington, MA) and maintained in specific pathogen-free-barrier animal facility approved by the American Association for Accreditation of Laboratory Animal Care. The mice were used for experiments at 7-8 weeks of age. Stable MDA-MB-435 cell variants expressing mutant Rac isoforms were harvested, resuspended in 1X sterile PBS, and injected subcutaneously into the mammary fat pad on the lower left side of the mouse at a concentration of $2x10^6$ per 100 μL. Tumors were measured with calipers once a week until the tumor reached 1.5 cm in diameter, or until the mouse became ill. The mouse was then euthanized in accordance with protocols approved by the Institutional Animal Care and Utilization Committee under guidelines from the Panel on Euthanasia, the American Association of Veterinary Medicine.

India Ink Lung Metastasis Assay

The lung was removed from the animal subsequent to euthanization and injected through the bronchus with a 15% India ink solution in PBS to saturation using a 28.5

gauge needle and 10 ml syringe. The lung was then suspended in Fekete's destaining solution as described in (Watts and Kennedy, 1998). The staining procedure results in a clear distinction between tumor foci (white) and normal lung tissue (black) on visual analysis. The lung tumors were quantitated and measured with calipers under a 4X dissecting scope (Bausch and Lomb).

4.3 Results

Characterization of mutant Rac stable cell lines.

All cell lines constructed are listed in Figure 4.1a. Low metastatic MDA-MB-435 variant MDA-MB-435Br1 was stably transfected with vector alone, myc-tagged Rac1(G12V), or myc-tagged Rac3(G12V). Highly metastatic MDA-MB-435 variant MDA-MB-435a6HG6 was stable transfected with vector alone, Rac1(T17N) or Rac3(T17N). For all of these cell lines, total Rac expression, as well as total Rac activity, was assayed. Total Rac protein expression is increased 2-fold in the stable MDA-MB-435Br1 dominant active mutants, as compared to the control. Moreover, Rac activity is greatly increased in the stable dominant active Rac mutant cell lines as compared to the vector control (Figure 4.1b). In the dominant negative Rac mutant stable cell lines, total Rac expression is again increased 2-fold over that of the control. However, total Rac activity is greatly decreased in the mutant cell lines as compared to that of the vector control (Figure 4.1c).

Dominant active Rac mutants increase cell cycle progression.

Active Rac proteins can signal to the cell cycle promoters JNK, p38MAP kinase, and NFkB (Cotteret and Chernoff, 2002). The ability of Rac to weakly transform cells is thought to be linked to these signal pathways (Westwick et al., 1997; van Leeuwen et al., 1995). Moreover, there is evidence to suggest that Rac3 is more efficient at promoting cell cycle progression than Rac1 (Mira et al., 2000). Therefore, we performed cell cycle analysis on the stable Rac mutant cell lines.

Subsequent to analysis, we found that dominant active Rac1 or Rac3 can increase the percentage of cells in S phase over that of the control, indicating an increase in cell cycle progression (Figure 4.2). However, dominant active Rac3 did not activate cell cycle progression more than dominant active Rac1, indicating little difference in the cell cycle promoters downstream of these two isoforms, or their ability to bind to them. Conversely, no difference was found between the percentage of cells in S phase of the dominant negative mutant stable cell lines and their control (Figure 4.3). Even though Rac proteins can activate cell cycle promoters, their endogenous interaction is weak and the signaling inefficient (Cotteret and Chernoff, 2002). Other proteins within the cell are better able to bind and active cell cycle signaling cascades, such as the map kinases signaling cascades (Cotteret and Chernoff, 2002). Therefore, blocking Rac activation has little effect on cell cycle progression *in vitro*, suggesting that the involvement of Rac in metastatic progression includes downstream effectors not involved in cell proliferation.

Ectopic Rac(G12V) expression augments the invasive phenotype of low metastatic breast cancer cells.

Invasive malignant cell morphology includes an increased number of focal adhesions, as well as an increase in actin structures such as cross-linked actin fibers and membrane ruffles (Condeelis et al., 2001). The morphology of the low metastatic cell variant MDA-MB-435Br1 when expressing vector alone is indicative of a less invasive cell. Actin fibers are not cross-linked, lamellipodia are limited to the proximal and distal ends of the cell, and focal adhesions are few (Figure 4.4). Conversely, MDA-MB-435Br1 cells expressing myc-Rac1(G12V) or myc-Rac3(G12V) exhibit cross-linked actin fibers, numerous focal adhesions, and lamellipodia expressed ubiquitously around the periphery of the cell (Figure 4.4). Expression of either myc-Rac1(T17N) or myc-Rac3(T17N) in the highly metastatic MDA-MB-435α6HG6 variant exhibit a less dramatic morphology than the dominant active mutants (Figure 4.5). Though, cells expressing Rac1(T17N) or Rac3(T17N) appear to exhibit smaller lamellipodia and less focal adhesions per cell than the vector control.

Rac mutants significantly alter cellular processes essential to metastatic behavior.

Because metastatic progression results from increased migration of malignant cells out of the basal lamina, subsequent adhesion to the extracellular matrix, and final invasion into distant tissues to establish secondary sites of metastasis, we measured the effect of Rac mutants on these processes *in vitro*. For each of these assays, cells expressed equal amounts of activated Rac1 or Rac3 mutant protein (Figure 4.1).

Recent data indicate that changes in cell adhesion play a critical role in tumor progression (Cavallaro and Christofori, 2004); thus, we tested the ability of Rac mutants to alter adhesive properties of malignant cells *in vitro*. Dominant active Rac1(G12V) or Rac3(G12V) cause a significant increase in adhesion to basal lamina when expressed in low metastatic MDA-MB-435Br1 as compared to the vector alone control, while dominant negative Rac1(T17N) or Rac3(T17N) cause a significant decrease in adhesion when expressed in high metastatic MDA-MB-435α6HG6 (Figure 4.6a, 4.7a).

A requirement of malignant cells to undergo metastasis is the acquisition of the ability to penetrate surrounding ECM proteins in order to migrate to distant tissues (Playford and Schaller, 2004); thus, we tested the effect of Rac mutants on both migration and invasion *in vitro*. Both myc-Rac1(G12V) and myc-Rac3(G12V) caused a significant increase in migration and invasion when expressed in the low metastatic variant (Figure 4.6b,c). Surprisingly, myc-Rac3(G12V) expressing cells invaded through basal lamina 1.5 times more than cells expressing Rac1(G12V) (Figure 4.6c). Invasion of high metastatic cells expressing dominant negative Rac1(T17N) or Rac3(T17N) was significantly diminished as compared to the vector alone control (Figure 4.7c). Furthermore, migration was also significantly reduced in highly metastatic cells expressing dominant negative mutants of Rac isoforms as compared to vector control (Figure 3.8c). Therefore, Rac activity is directly involved and necessary for increased migration during the invasion of metastatic breast cancer cells. Taken together, this data establishes the efficacy both Rac1 and Rac3 in metastatic processes.

Rac mutants alter pulmonary metastasis *in vivo.*

The mouse model of experimental metastasis is an assay used to determine the effects of stable cell lines on the process of metastasis *in vivo*. Once injected into the mammary fat pad of immunocompromised mice, human mammary cancer cells must form a primary tumor, migrate away from the primary tumor, travel through the blood stream, exit the blood stream and form a secondary, metastatic, tumor at a distant site. Therefore, the stable cell lines expressing mutant forms of Rac isoforms, as well as their vector controls, were tested in this model.

Injection of stable cell lines expressing dominant active forms of Rac1 or Rac3 promoted pulmonary metastasis as compared to the control. Once the primary tumors had reached 1-1.5 cm in diameter, the lungs were assayed for pulmonary metastases. The vector alone control cell lines MDA-MB-435Br1 never exhibited pulmonary metastasis, as the excised lungs demonstrated no lesions in any of the lobes (Figure 4.8a). Both of the mutant Rac cell lines were able to contribute to lung metastases, as the excised lungs exhibited numerous lesions in all three lobes (Figure 4.8b,c). Subsequent to quantitation, MDA-MB-435Br1Rac1(G12V) exhibited an average of 28 pulmonary lesions, with an average volume of 3.6 mm^3. MDA-MB-435Br1Rac3(G12V) exhibited an average of 14 pulmonary lesions, with an average volume of 1.71 mm^3. Therefore, both Rac1 and Rac3 can contribute to breast cancer metastasis *in vivo*.

Injection of stable cell lines expressing dominant negative Rac1 or Rac3 blocked pulmonary metastasis as compared to the vector alone control (Figure 4.9). Moreover, dominant negative Rac1 or dominant negative Rac3 appeared to slow the primary tumor

growth *in vivo* (Figure 4.9). Primary tumors formed by the injection of cell lines expressing Dominant negative Rac1 averaged 50 mm^3, while those formed by the injection of cell lines expressing dominant negative Rac3 averaged 100 mm^3. The average primary tumor formed by the vector alone control was 300 mm^3. Furthermore the vector alone control, MDA-MB-435a65HG6, exhibited several pulmonary metastases, while the dominant negative Rac isoforms did not (Figure 4.9). On average, the vector alone control exhibited 6 pulmonary lesions, with an average volume of 3.6 mm^3.

4.4 Discussion

In this study we demonstrate, for the first time, the efficacy of both the Rac1 and Rac3 isoforms in the malignant progression of human breast cancer. Because Rac1 and Rac3 both have been implicated in breast cancer (Leung et al., 2003; Bouzahzah et al., 2001), we carried out a comparative study between the two isoforms. Activation of the Rac1 or Rac3 isoforms in a transformed cell with a non-invasive morphology drastically changes the invasive actin structures and increases the number of focal adhesions. Activation of Rac1 or Rac3 also causes an increase in cell cycle progression in low metastatic breast cancer cells. Additionally, we found that blocking Rac activity by expressing dominant negative mutations of Rac1 or Rac3 significantly curtailed cellular processes critical for metastatic progression *in vitro*. Moreover, we found that augmenting endogenous Rac activity by expressing dominant active Rac1 or Rac3 led to

a significant increase in adhesion, migration, and invasion. Taken together, these data substantiate not only a vital role for Rac1 in breast cancer metastasis, but also a vital role for Rac3, for the first time. In fact, expression of a dominant active Rac3 in the MDA-MB-435Br1 low metastatic cell variant increased invasion through basal lamina 1.5 times as compared to expression of dominant active Rac1. This difference suggests an enhanced ability of the cells expressing Rac3(G12V) to degrade the extracellular matrix, allowing for invasion, as compared to the cells expressing Rac1(G12V). It is possible that Rac3 is more efficient at activating proteins that degrade extracellular matrix proteins, or matrix metalloproteinases (MMPs), than is Rac1. Because our *in vitro* data was convincing of a role for Rac3 in human breast cancer metastasis, we took an *in vivo* model approach: the nude mouse model of experimental metastasis.

Justification of the Nude Mouse Model of Experimental Metastasis

In vitro assays modeling invasion, adhesion, and migration are extremely limiting in their ability to mimic all aspects of metastatic progression. Recently, the idea of the tissue surrounding tumor cells, or the tumor microenvironment, playing a decisive role in triggering invasion has begun to receive increased attention (Quaranta and Gianelli, 2003). These *in vitro* assays that measure individual aspects of cell invasion fail to include the tumor microenvironment, which can contribute substantially to the metastatic process. The only way to mimic the tumor microenvironment is to use an animal model that closely shares human characteristics (Khanna and Hunter, 2005). The mouse model of experimental metastasis is the most common *in vivo* model used to mimic human

cancer progression. This model is the closest mimic to human cancer progression because it includes the delivery of cancer cells to the anatomic location or tissue from with the tumor was derived (orthotopic transplantation). Orthotopic transplantation has been shown to result in tumor models that more closely resemble human cancers with respect to tumor histology, vascularity, gene expression, responsiveness to chemotherapy, and metastatic biology (Bibby, 2004; Khanna, et al., 2000).

However, there are limitations to this model. For example, tumorigenesis is not only the result of uncontrolled proliferation of a mutated cell, but it is a complex interaction between the tumoregenic tissue and the environmental tissue in which it arises (Quaranta and Gianelli, 2004). In our system, we are implanting human cells in mouse tissue. Because of the discrepancy between the two species, this tumor implantation may not recapitulate all interactions between the neoplastic cells and tumor microenvironment essential to the process of human tumor dissemination. Additionally, mechanical disruption of the area affected by the implantation itself may permit tumor cells to disseminate directly into the circulatory system, bypassing invasion into the surrounding tissue altogether (Khanna and Hunter, 2005). Finally, orthotopic injection of genetically engineered cells into the mouse model is limited by its reliance on cultured cells. Cultured cells used in spontaneous mouse models of cancer have been adapted for years to grow in two-dimensional matrix platforms, which create adaptations that are foreign to a three-dimensional system (Khanna and Hunter, 2005). The adaptations that allow cells to grow in culture may alter the pathways by which endogenously arising metastasis survive. Clearly, the mouse models have their limitations. Therefore, a multi-faceted

approach to studying metastatic progression, one which includes an *in vitro* component as well as an *in vivo* approach, was taken.

In vivo Data from Animal Models

However, when comparing Rac1 with Rac3 in the animal models, it appears that Rac1 is more efficient at promoting metastasis than is Rac3. The cell lines expressing a dominant active Rac1 promoted the formation of more pulmonary metastases with larger volumes than the cell line expressing a dominant active Rac3. Moreover, blocking Rac activity caused smaller primary tumors to form in the marine mammary fat pad than blocking Rac3. This result is in contrast to our cell cycle data, which showed no difference in cell cycle progression between the cell line blocking Rac1 activity and the cell line blocking Rac3 in culture. Perhaps the murine model is a better measure of how cells will behave *in vivo*. Cells encounter a much different, 3-dimensional environment *in vivo* than *in vitro*. More and more data is beginning to indicate that the tumor microenvironment is equally as important as the cellular make up of the tumor in predicting invasive behavior (Quaranta and Giannelli, 2003). In addition to responding to the composition of the substratum, cells sense and react to physical properties that include 3-dimensionality and the rigidity of the matrix (Yamada et al., 2003). Moreover, the molecular composition of focal adhesions that cells form in 3-dimensional matrices are very different than those formed in more traditional, 2-dimensional matrices (Cukierman et al., 2001; Yamada et al., 2003). Additionally, cells show more rapid morphological changes, migration, and proliferation in 3-dimenstional matrices compared

to standard 2-dimensional matrices (Yamada et al., 2003). Therefore, models that mimic the true microenvironment are becoming increasingly important when trying to predict the outcome of tumor invasion.

Our *in vivo* data point to the conclusion that Rac1 is more efficient at both promoting growth and increasing metastasis in the murine model. Rac1 and Rac3 differ in their C-terminus region which is essential for subcellular localization (Haataja et al., 1997). Even though protein function is likely partially redundant due to the homology of the downstream effector loops, these proteins have been found to differ in their localization within certain types of cells (Bolis et al., 2003). Differential subcellular localization can place proteins in the proximity of different signaling cascades, resulting in differential function. However, more experiments are needed to show that Rac1 actually acts differently than Rac3 with respect to human breast cancer. For example, creating stable knockdowns with siRNA would be an extremely valuable tool to elucidate the functions of Rac3 that differ from the functions of Rac1. By using dominant active mutations of isoforms that are so closely related, the possibility that downstream effectors are cross-activated is very likely. The same is true for the dominant negative mutants. Our approaches in this research are somewhat limited, and there are many future experiments that should be considered with regard to differential Rac1 and Rac3 function.

Conclusion

In conclusion, our data strongly suggests that both Rac1 and Rac3 are important for the metastatic phenotype of human breast cancer. By using variants of the same cell line, we have minimized genetic variation. Because of the similarity of the genetic background of these variants, Rac activity differences were striking, and very suggestive of an essential role for this subfamily of proteins in the metastatic progression of human breast cancer.

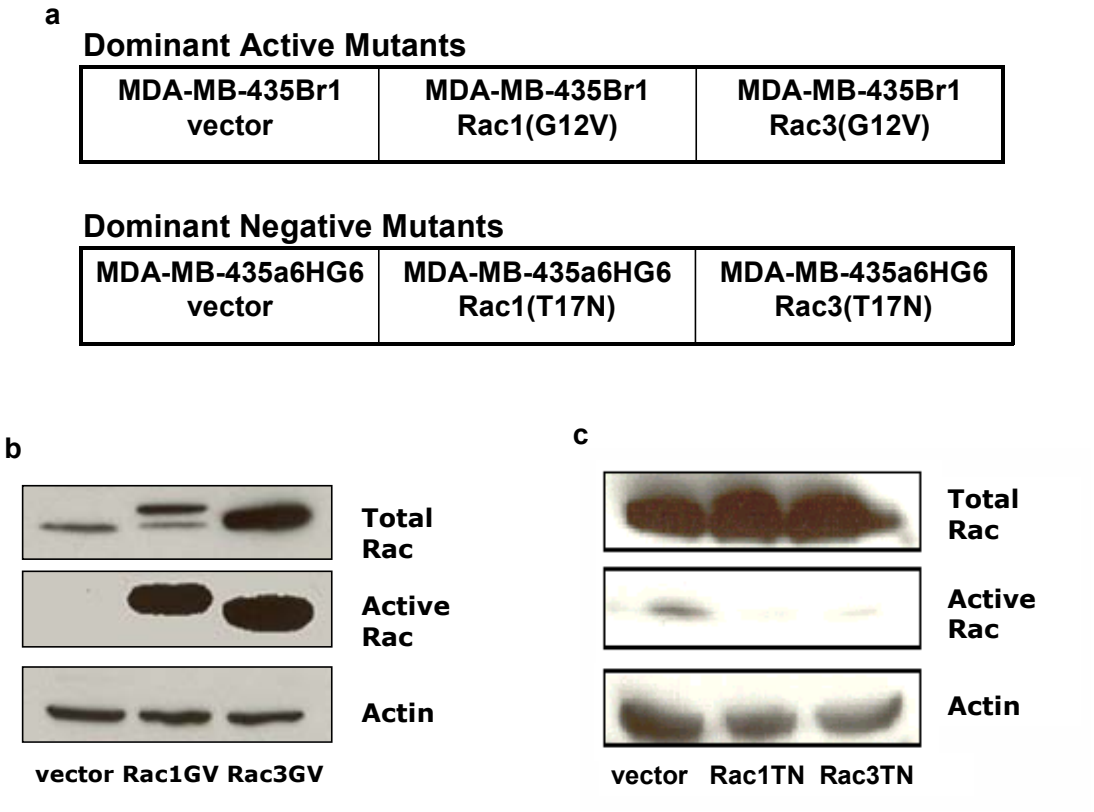
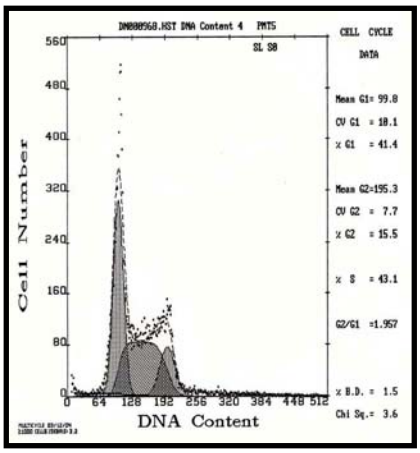
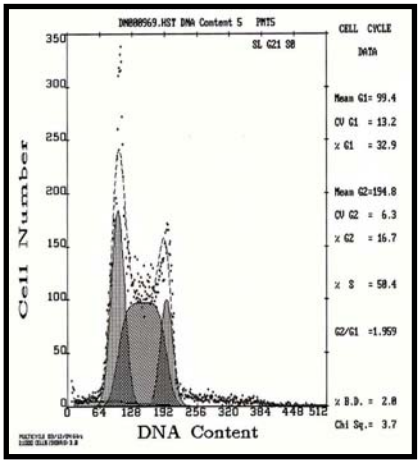


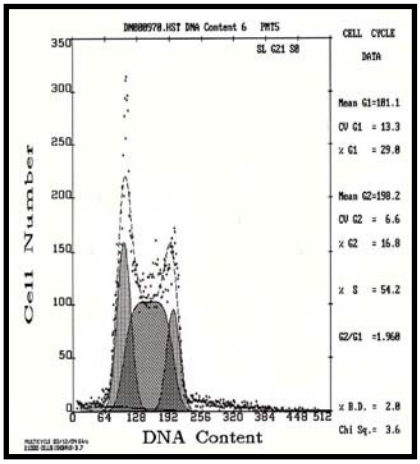
Figure 4.1. Characterization of stable cell lines expressing mutant Rac isoforms. Whole cell lysates of all stable cell lines **(a)** were subjected to SDS-PAGE followed by western blot analysis for total Rac using an anti-Rac antibody. Dominant active cell lines are shown in **(b)**, dominant negative cell lines are shown in **(c)**. Rac activity was assayed using the PAK-PBD activity assay. Equal loading of lanes was maintained by performing a total protein assay and is confirmed by western blot analysis for total actin. Results are representative of three to five independent experiments.



Vector control



Rac1(G12V)



Rac3(G12V)

Figure 4.2 Cell cycle analysis of stable cell lines expressing vector alone, dominant active Rac1, or dominant active Rac3. Vector alone **(a)**, dominant active Rac1 **(b)**, or dominant active Rac3 **(c)** stable cell lines were fixed, stained with PI, and subjected to flow cytometry. S phase is the peak between G1 and G2 phases, and is representative of cell cycle progression. MDA-MB-435Br1 Vector alone S phase is 43.5% of all cells assayed; MDA-MB-435Br1Rac1(GV) S phase is 50.4% of all cells assayed; MDA-MB-435Br1Rac3(GV) S phase is 54.2% of all cells assayed.

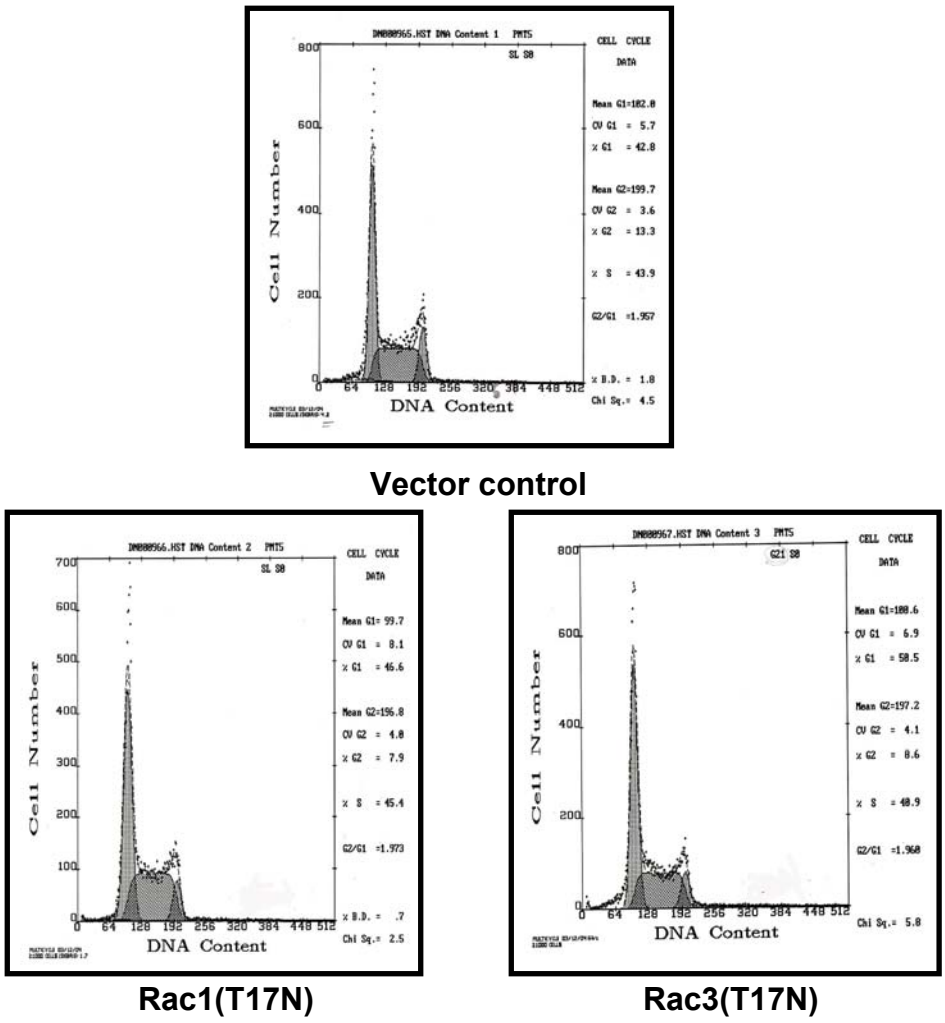


Figure 4.3 Cell cycle analysis of stable cell lines expressing vector alone, dominant negative Rac1, or dominant negative Rac3. Vector alone **(a)**, dominant negative Rac1 **(b)**, or dominant negative Rac3 **(c)** stable cell lines were fixed, stained with PI, and subjected to flow cytometry. S phase is represented by the peak between G1 and G2 phases, and is representative of cell cycle progression. MDA-MB-435a6HG6 vector alone S phase is 43.9% of all cells assayed; MDA-MB-435a6HG6Rac1(TN) S phase is 45.4% of all cells assayed; MDA-MB-435a6HG6Rac3(TN) S phase is 40.9% of all cells assayed.

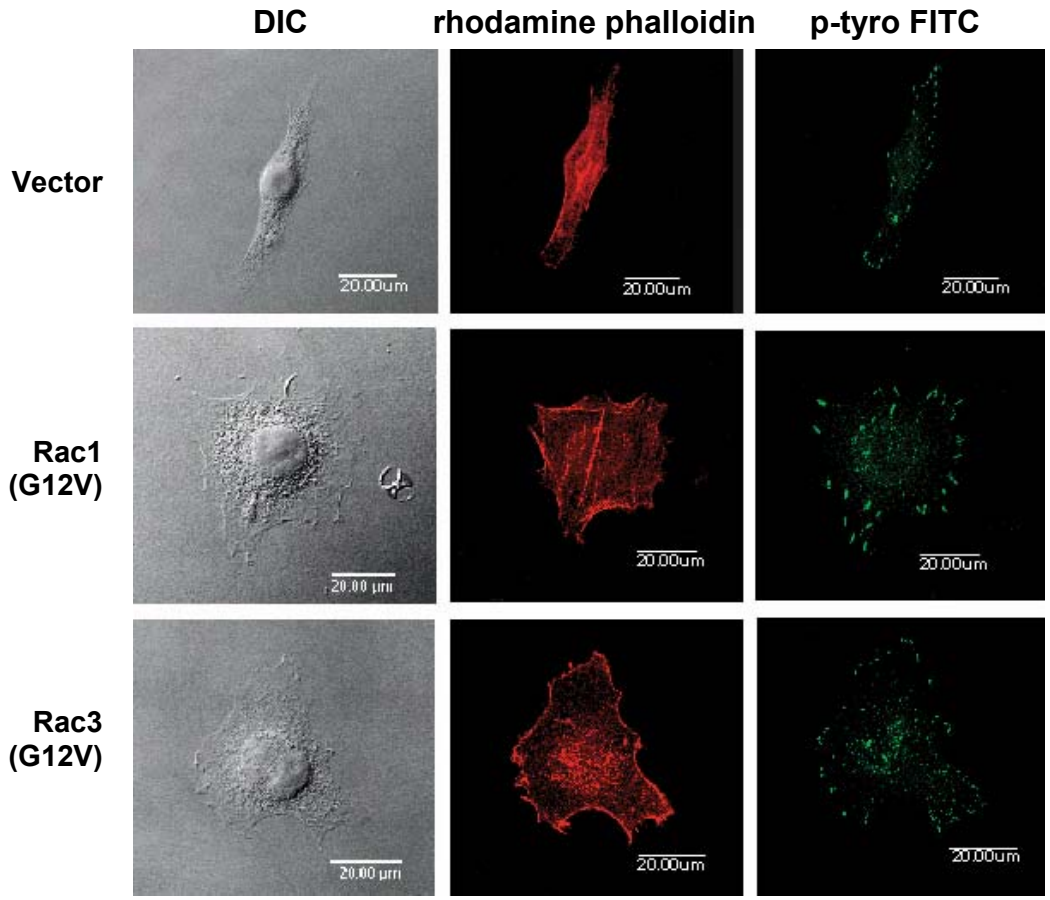


Figure 4.4. Effects of ectopic dominant active Rac(G12V) expression in low metastatic variant MDA-MB-435Br1 on cellular morphology. Confocal DIC and fluorescent microscopy were performed on MDA-MB-435Br1 cell variant stably expressing vector alone, myc-Rac1(G12V), or myc-Rac3(G12V). Cells were plated on glass coverslips, fixed in 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Actin was then visualized with rhodamine phalloidin and focal adhesions were visualized with an anti-p-tyro antibody followed by an FITC conjugate.

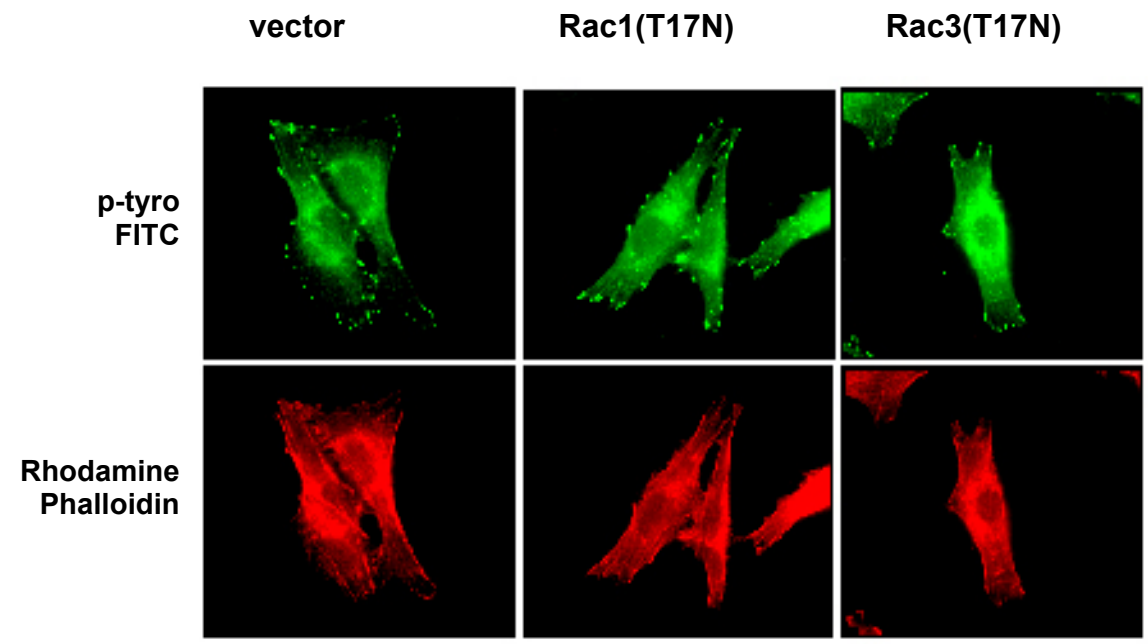


Figure 4.5. Effects of ectopic dominant negative Rac(T17N) expression in highly metastatic variant MDA-MB-435a6HG6 on cellular morphology. Fluorescent microscopy was performed on the MDA-MB-435a6HG6 cell variant stably expressing vector alone, myc-Rac1(T17N), or myc-Rac3(T17N). Cells were plated on glass coverslips, fixed in 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Actin was then visualized with rhodamine phalloidin and focal adhesions were visualized with an anti-p-tyro antibody followed by an FITC conjugate.

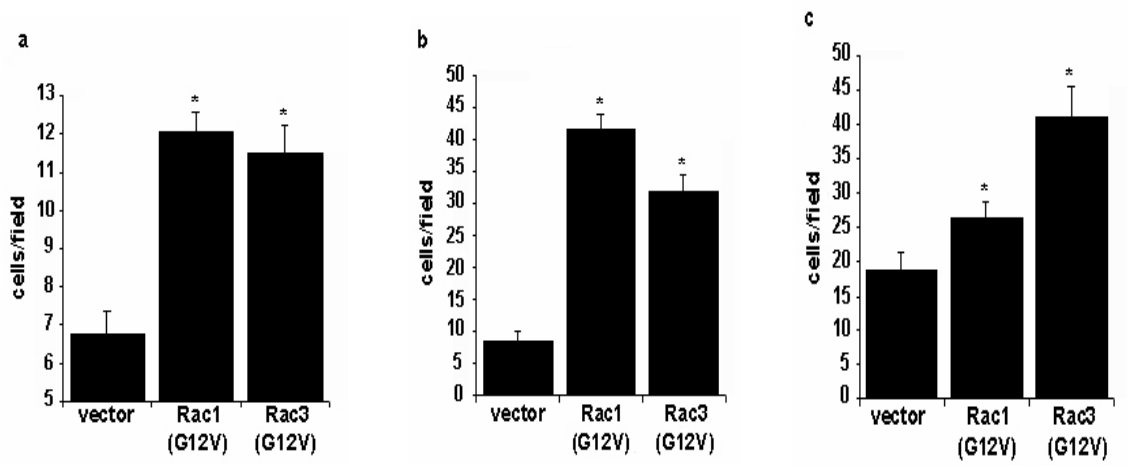


Figure 4.6. Effects of dominant active Rac isoforms on metastatic properties, as measured *in vitro*. MDA-MB-435Br1 cells expressing vector alone, myc-Rac1(G12V), or myc-Rac3(G12V), were subjected to adhesion **(a)**, haptotaxis **(b)**, and invasion **(c)** assays. Cells were counted under (200X) for adhesions assays, and (400X) for haptotaxis and invasion assays. Y-axis represents the number of cells/field for at least 20 microscopic fields per cell line. Bars represent standard error of the mean, and is representative of at least 3 separate experiments. An asterix indicates a statistically significant difference compared to the control, vector alone, as determined by a Student's *t*-test ($P<0.05$).

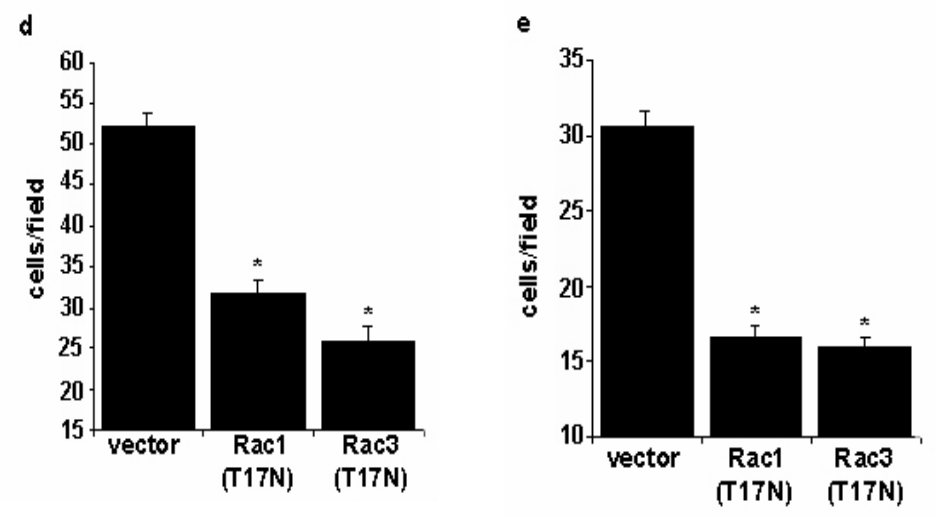


Figure 4.7. Effects of dominant negative Rac isoforms on metastatic properties, as measured *in vitro.* MDA-MB-435α6HG6 cells transiently expressing vector alone, myc-Rac1(T17N), or myc-Rac3(T17N), were subjected to adhesion **(d)**, and invasion assays **(e)**. Cells were counted at (200x) for adhesions assays, and (400x) for haptotaxis and invasion assays. Y-axis represents the number of cells/field for at least 20 microscopic fields per variant. Bars represent standard error of the mean, and is representative of at least 3 separate experiments. An asterix indicates a statistically significant difference compared to the control, vector alone, as determined by a Student's *t*-test ($P<0.05$). (Migration of cells expressing dominant negative Rac isoforms was shown in Chapter 3, figure 3.8.)

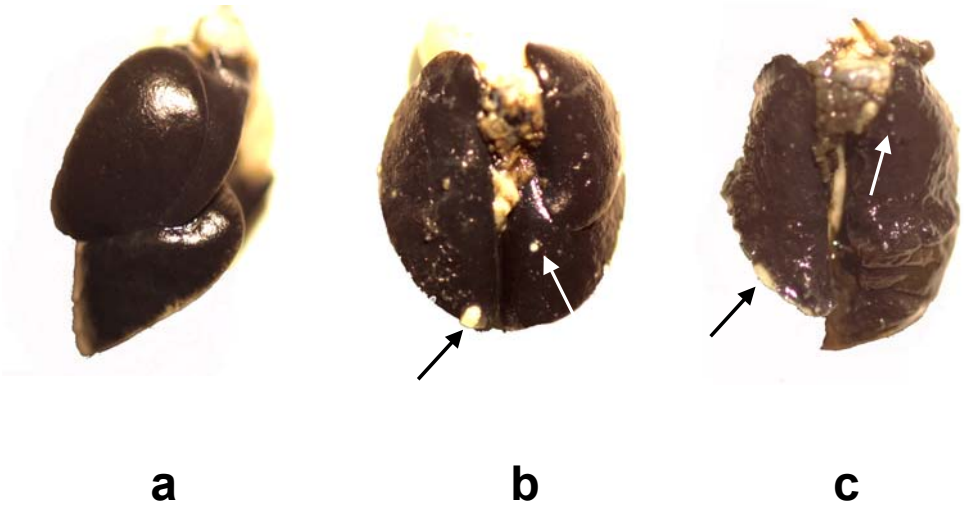


Figure 4.8. Effects of dominant active Rac isoforms on pulmonary metastasis, as measured *in vivo.* Representative appearance of murine lungs where metastasized colonies are visible as white foci 3-4 months subsequent to fat pad injection. **(a)** MDA-MB-435Br1 vector control, **(b)** MDA-MB-435Rac1(G12V), **(c)** MDA-MB-435Rac3(G12V). Arrows indicate sites of distant metastasis.

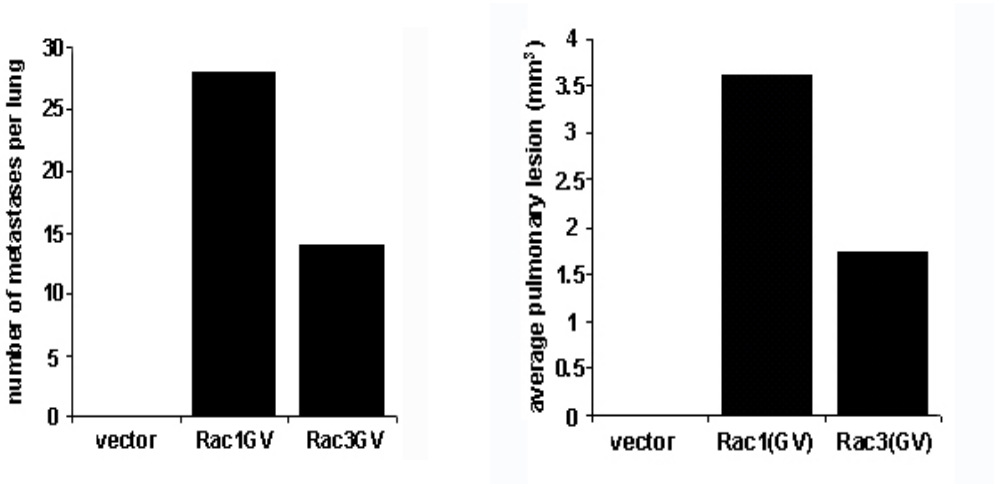


Figure 4.9. Effects of dominant active Rac isoforms on pulmonary metastasis, as measured *in vivo.* **(a)** Lesion number is lungs was counted, and Rac(G12V) expressing cells show an increase in colony formation. **(b)** Average pulmonary lesion size, as measured under a 4X dissecting scope with calipers.

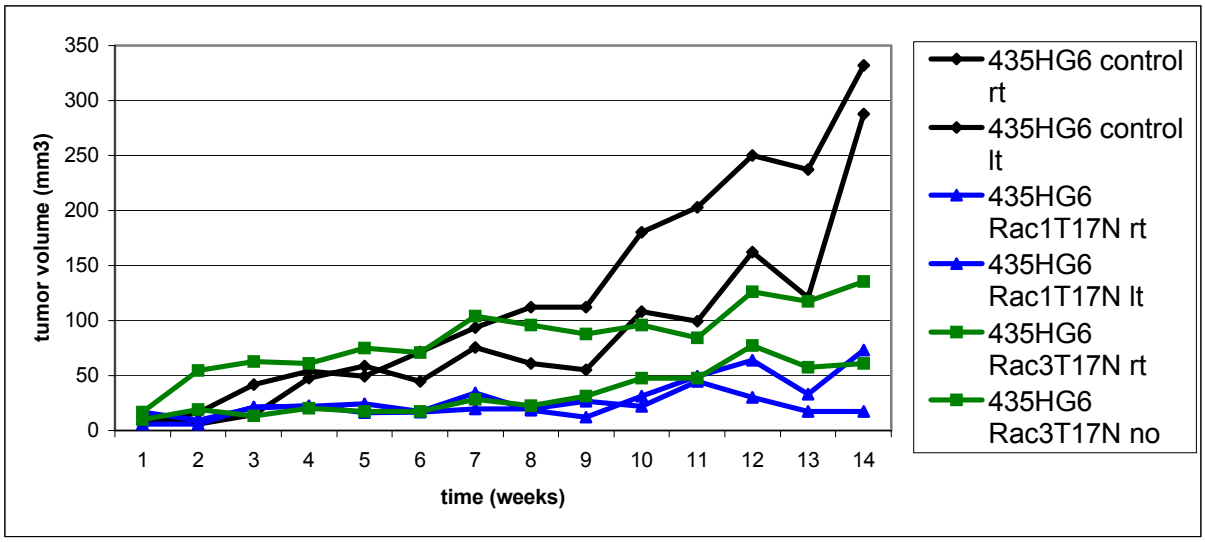


Figure 4.10. Effects of dominant negative Rac isoforms on primary tumor size, as measured *in vivo.* Cell lines expressing dominant negative Rac constructs reduced the size of the primary tumor formed, as compared to vector alone 15 weeks subsequent to mammary fat pad injection.

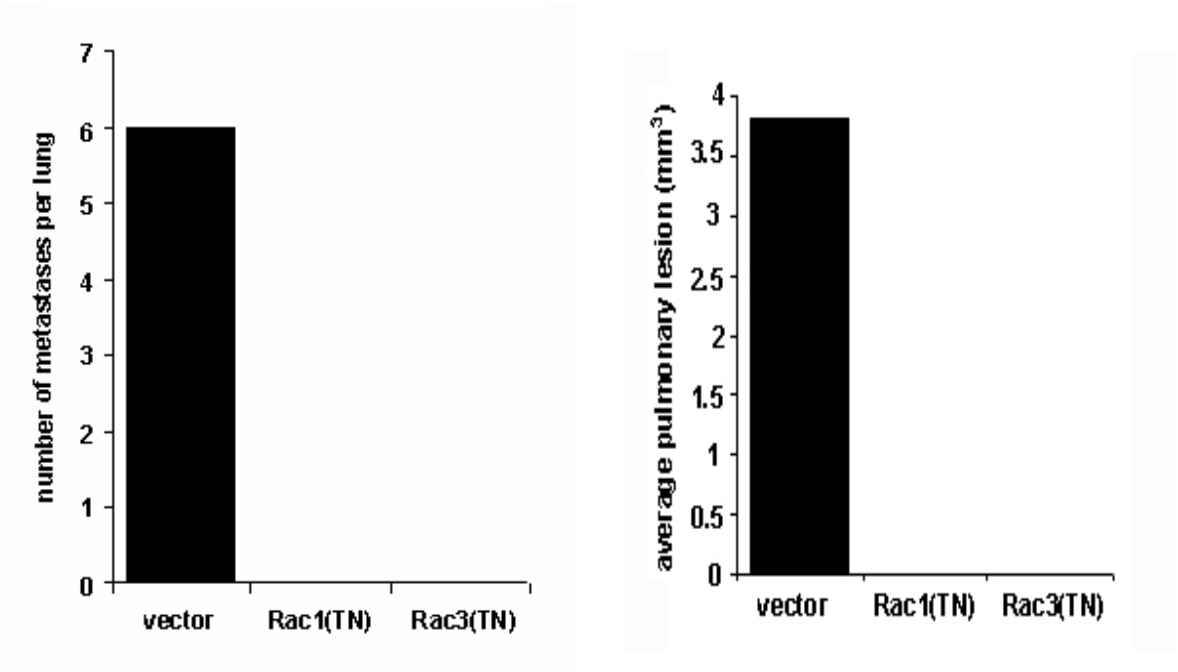


Figure 4.11. Effects of cells expressing dominant negative Rac isoforms on pulmonary metastasis, as measured *in vivo.* (a) Cell lines expressing dominant negative Rac 1 or 3 constructs blocked metastatic lung tumor formation, as compared to the vector control 15 weeks subsequent to fat pad injection. **(b)** Average pulmonary lesion size, as measured under a 4X dissecting scope with calipers.

5. Conclusions and Future Experiments

5.1 Conclusions

Overview: Metastasis Regulators in Human Breast Cancer

The foremost goal of the research presented here is the elucidation of proteins capable of either inducing or negatively regulating breast cancer metastasis. As mentioned earlier, cancer not only presents an interesting molecular mechanism challenge, but it also presents a terrible and debilitating disease. Furthermore, cancer has seen less success in the advancement of treatment over the past 50 years than heart disease, cerebrovascular disease, and pneumonia, diseases which are considered the most deadly diseases in the US (ACS, 2005). Breast cancer is the leading type of cancer occurring in women in the US, and it is estimated that one in eight women will develop breast cancer, and of these women, 30% will die from metastatic progression (Bowcock, 1999). Therefore, it is essential to elucidate the mechanisms by which breast cancer metastasizes in order to prevent further mortality.

To date, there are very few *bona fide* "metastasis suppressors", and even fewer metastasis suppressors specifically related to breast cancer (Keller, 2004). Of these genes regarded as possible metastasis suppressors in breast cancer, few have been thoroughly investigated. Most evidence is based on expression patterns in non-invasive versus invasive tissue samples from breast biopsies and tissue aspirations (Jiang et al., 2004; Steeg et al., 2003). Our approach is to use this data, but apply techniques of protein

biochemistry and cell biology to elucidate the mechanisms of the candidate proteins in order to pinpoint specific protein interactions that inhibit or upregulate breast cancer metastasis. The prevalent thought in the field of metastasis suppressors is that an improved molecular and biochemical understanding of the metastatic process is expected to fuel the development of new therapeutic approaches (Steeg et al., 2003). These new therapeutic approaches are, unfortunately, sorely needed.

The idea of metastasis suppressors as therapeutic targets could involve the restoration of a metastasis suppressor gene or the inhibition of a metastasis inducer gene, to the extent that it could interrupt a facet of the metastatic cascade and produce a clinical benefit. Anti-cancer drug development is currently based on *in vivo* models of tumorigenecity, including assays with immunocompromised mice. Our approach is to use current models of metastasis assays with immunocompromised mice to demonstrate the efficacy of candidate metastasis suppressors or metastasis inducers *in vivo*. Our hope is that the information presented in this research can be used to develop novel treatments for patients with metastatic breast cancer.

Recent data indicates strongly that growth at a primary tumor site and growth at a metastatic site differ by the expression and/or context-dependent function of the metastasis regulator, and that a wide variety of signaling pathways are affected (Steeg et al., 2003). Data presented here argues a strong case for PTEN as a metastasis suppressor and Rac proteins as metastasis inhibitors, for the fact that they fit the criteria. PTEN expression has been shown to be inversely correlated with increasing metastatic potential in a variety of human breast tumors (Lee et al., 2004). Furthermore, PTEN negatively

regulates PIP3. PIP3 is a second messenger that regulates a myriad of signaling pathways, including those involved in cell survival, cell cycle progression, as well as cell migration and invasion (Rameh and Cantley, 1999). Additionally, Rac expression has been shown to be correlated with increasing metastatic potential in a variety of human tumors (Fritz et al., 1999). Rac activation can also regulate numerous signaling pathways, including those involved in cell survival, cell cycle progression, as well as cell migration and invasion (Etienne-Manneville and Hall, 2002). Our data makes a convincing case that PTEN curtails migration in metastatic human breast cancer cells. Our data also demonstrates that in an *in vivo* model, Rac proteins can promote tumor metastasis when activated, and block tumor metastasis when inhibited. Taken together, these data support the usefulness of the PTEN protein and the Rac proteins in possible metastasis therapeutics for breast cancer.

Direct Implication of Rho GTPases in Metastatic Human Breast Cancer

The data presented here, for the first time, directly implicate the Rho GTPases Rho, and Rac in the progression of human breast cancer metastasis. A range of cell variants, varying in their metastatic potential to metastasize in the nude mouse model of experimental metastasis, were derived from the same parental cell lines, MDA-MB-435 (Mukhopadhyay et al., 1999). We used this panel of metastatic variants to identify differential protein expression and activity. This panel gives us a powerful tool to study the regulators of metastasis in that all cell included in the panel have very similar genetic profiles. Most studies before using a "range" of cell lines differing in metastatic potential

have used cell lines cloned at different times from different patients. This variety introduces errors, due to extensive differences in the genetic profiles of these cells. Because our panel was derived from the same parental line, we have a powerful tool to study those proteins both causal and inhibitory specifically in relation to breast cancer metastasis.

It is generally agreed that an improved molecular and biochemical understanding of the metastatic process is expected to fuel the development of new therapeutic approaches (Steeg et al., 2003). Recent data indicate strongly that growth at a primary tumor site and growth at a metastatic site differ by the expression and/or context-dependent function of the metastasis regulator (Steeg et al., 2003). Additionally, proteins identified as metastasis regulators would be involved in numerous signaling pathways, including those important for primary tumorigenesis, as well as metastatic progression. Rho GTPases fit all of these criteria. Rac and Cdc42 GTPases regulate motility via PAK, and Arp2/3 via WASP and WAVE (Etienne-Manneville and Hall, 2002). These proteins also regulate cell cycle progression and cell survival via NFkB, MEKK, MLK, as well as stress-activated p38 MAP kinases (Cotteret and Chernoff, 2002). In fact, Rac3 was found to have a significant impact on the proliferation of breast cancer cells (Mira et al., 2000). RhoA and C proteins also regulate motility via mDia (stress fiber formation) and ROCK (acto-myosin contraction). Therefore, Rho GTPases become ideal candidates for identification as “metastasis regulators”.

Direct role for Rac3 in metastatic human breast cancer

The data presented here, for the first time, directly implicate the recently cloned Rho GTPase protein Rac3 in the metastatic progression of human breast cancer. A comparative study between two isoforms of Rac, Rac1 and Rac3, was carried out due to the fact that both isoforms have been implicated in breast cancer tumorigenesis (Leung et al., 2003; Bouzahzah et al., 2001). What has not been addressed, however, is the possibility of a role for Rac3 in breast cancer metastasis. Recent data has shown a specific role for Rac3 in the hyperproliferation of breast cancer cells, as well as the ability of Rac3 to promote primary mammary lesions in mice (Mira et al., 2000; Leung et al., 2003). The study presented here, however, is novel in that we substantiate a role for Rac3 in human breast cancer progression to the metastatic state.

In summary, we found that blocking Rac activity by expressing dominant negative mutations of Rac1 or Rac3 significantly curtailed cellular processes critical for metastatic progression *in vitro*. Moreover, we found that augmenting endogenous Rac activity by expressing dominant active Rac1 or Rac3 led to a significant increase in adhesion, migration, and invasion. Taken together, these data substantiate not only a vital role for Rac1 in breast cancer metastasis, but also a vital role for Rac3, for the first time. In fact, expression of a dominant active Rac3 in the MDA-MB-435Br1 low metastatic cell variant increased invasion through basal lamina 1.5 times as compared to expression of dominant active Rac1. This difference suggests an enhanced ability of the cells expressing Rac3(G12V) to degrade the extracellular matrix, allowing for invasion, as compared to the cells expressing Rac1(G12V). It is possible that Rac3 is more

efficient at activating proteins that degrade extracellular matrix proteins, or matrix metalloproteinases (MMPs), than is Rac1. Most significantly, however, we found that blocking Rac3 activation could block metastasis in an *in vivo* model. Additionally, we found that activating Rac3 could promote metastasis in an *in vivo* model. Taken together, these data cement a significant role for the Rac isoform Rac3 in human breast cancer progression.

5.2 Future Experiments

Investigation of a Role for RhoC in Breast Cancer Metastasis

The RhoA and RhoC genes are 92% identical (Ridley, 1997). They are regulated in the same GDP/GTP cycle, and can be sequestered in the cytosol by RhoGDIs (Wheeler and Ridley, 2004). No clear difference of the RhoGEFs Vav, p115RhoGEF, and Brc, or the Rho GAP p190RhoGAP, in binding affinity between the two proteins (Wheeler and Ridley, 2004). RhoA and RhoC have both been found to localize either in the cytosol or the plasma membrane (Wheeler and Ridley, 2004). Rho effector proteins such as ROCK, mDia, Rhotekin, Rhophillin, and Citron Kinase have been found to interact with both isoforms (Wheeler and Ridley, 2004). These findings suggest that there will be little difference between RhoA and RhoC function *in vivo*.

However, RhoA and RhoC do exhibit slight differences in sequence, which translate into substantial differences in function (Wheeler and Ridley, 2004). Most divergence between the protein sequences is found at the C-terminus, but some

variability is found in the insert loop (Wheeler and Ridley, 2004). These differences would indicate a difference in localization, and perhaps a difference (although not spectacular) in binding affinity to GTPase regulators. While RhoA and RhoC expression have both been found to be upregulated in certain tumors, it appears that only RhoA can promote transformation of cultured fibroblasts (Wheeler and Ridley, 2004). Recently, though, RhoC has attracted substantial interest with its increased expression being correlated to increased invasion in several types of cancers: gastric, bladder, colon, breast, melanoma, and non-small-cell lung carcinoma (van Golen et al., 2000; Clark et al., 2000; Kamai et al., 2003; Shikada et al., 2003; Kondo et al., 2004; Frtiz et al., 1999). Other evidence indicates that RhoA impedes, while RhoC stimulates invasion (Simpson et al., 2004). This difference could be due to the finding that RhoC binds with more affinity to ROCK than does RhoA (Sahai and Marshall, 2002). ROCK is an important downstream effector of Rho proteins that activates cell contraction via phosphorylation and activation of myosin light chain (Riento and Ridley, 2003). More contraction could lead to enhanced motility. Additionally, RhoC siRNA has been shown to block breast cancer metastasis *in vivo* in the mouse model of metastasis (Pille et al., 2005).

Herein, we show convincing evidence that RhoC plays an important role in the invasive and metastatic capability of human breast cancer progression. RhoC is more highly expressed in the more metastatic cell variants, while RhoA expression is relatively equal. Additionally, blocking RhoA has no effect on cell migration in the most migratory cell variant (MDA-MB-435α6HG6). Future experiments include cloning the RhoC cDNA into a mammalian expression vector with antibiotic selection markers and site

directed mutagenesis to create the dominant negative mutant. Subsequent to the generation of stable cell lines, analysis of blocking RhoC and the affect of that blockage on motility and invasion will be analyzed. Finally, we would like to use the nude mouse model of experimental metastasis to test out hypothesis *in vivo*.

Cross Talk between Rho GTPases in breast cancer

Rho GTPases have long been known to participate in cross talk (Burridge and Wennerberg, 2004). However, the extent to which they do this and the implied physiological significance is still under investigation. The activation of the Rho family of small GTPases, namely Rho, Rac, and Cdc42, is a critical event in the integrin-mediated regulation of the cellular processes of adhesion, migration, and invasion (Miranti and Brugge, 2002; Hynes, 2002). During these processes, crosstalk between the Rho GTPases, their isoforms, and their downstream effectors are coordinated in a highly complex and not completely understood manner (Schmitz et al., 2000). Activation of appropriate levels, together with temporal and spatial coordination, must be precisely regulated in order to achieve normal adhesion and motility (Price and Collard, 2001). The balance between Rho, Rac, and Cdc42, as well as the localized activity of these proteins, is essential for the determination of cellular morphology and invasive behavior (Evers et al., 2000).

A recently published study revealed a compensatory relationship between RhoA and RhoC at both expression and activation levels, and a reciprocal relationship between RhoA and Rac1 activation (Simpson et al., 2004). This finding implies that one tumor

marker or metastasis marker is not enough to predict the outcome of tumor invasion. For example, increased RhoA expression but decreased RhoC expression may indicate a tumor that is not extremely aggressive, while a tumor expressing elevated RhoC but decreased RhoA might present a more invasive phenotype.

Because we see elevated Rac activity and RhoC expression in the most highly metastatic variant, it would be interesting to investigate the interplay between these proteins. I would hypothesize that expressing RhoA in the highly metastatic cell line would decrease invasiveness, which is a result contradictory to classical hypotheses and approaches. Additionally, exploring the crosstalk between Rac3 and RhoC would be completely novel, based on the finding that Rac3 is important in cell proliferation (Mira et al., 2000). Specifically, the approach to study crosstalk would rely on double mutant studies. That is, expression of a dominant active RhoA and a dominant negative RhoC. Hypothetically, this expression would decrease both cell motility and Rac activation, while the reciprocal experiment (expression of dominant negative RhoA and dominant active RhoC), would result in increased motility and increased Rac activation. It may become important in the future to know the mechanisms of crosstalk, for they may be useful to predict patient outcome.

Elucidation of Rac1 versus Rac3-specific GEFs

Rac1 and Rac3 are 92% homologous (Haataja et al., 1997). Yet, why would two proteins exist to function in exactly the same way in cells? Some differences have been noted in expression and function between the two isoforms. For example, Rac3 has been

found to be more highly expressed in neural tissue than is Rac1 (Bolis et al., 2003). This differential distribution is thought to support a role for Rac3 specifically in the remodeling of Purkinje cell neuritic terminals at the time of synaptogenesis (Bolis et al., 2003). Rac3 has been found to interact with the integrin-binding protein CIB (calcium and integrin-binding), a protein with which neither Rac1 nor Rac2 interact (Haataja et al., 2002). This differential binding is thought to implicate Rac3 specifically in integrin-associated cytoskeletal reorganization during αIIBβ3-mediated adhesion (Haataja et al., 2002). Furthermore, Rac3, but not Rac1, was found to control proliferation in breast cancer cells (Mira et al., 2000).

However, many similarities have also been noted. The effector binding region in the Rac-like GTPases, or the switch region, is found to be 100% identical in Rac1 and Rac3 (Haataja et al., 1997). This finding would indicate that Rac1 and Rac3 would bind the exact same downstream effectors. Comparative functional analysis of the Rac GTPases indeed revealed that Rac1 and Rac3 exhibit consistent biochemical characteristics such as GTP hydrolysis and effector binding, and exhibited the same binding affinity for PAK (Haeusler et al., 2003). Furthermore, studies that addressed Rho GTPase effect on the organization of the cytoskeleton found that expression of both Rac1 and Rac3 resulted in the formation of lamellipodia (Aspenstrom et al., 2004). However, these proteins differ in the amino acid sequence of their C-terminus, and are thus differentially prenylated (Joyce and Cox, 2003). Differential prenylation indicates differential localization. Rac1 and Rac3 also differ in their insert regions, which

influence interaction with guanine nucleotide exchange factors (GEFs) (Mira et al., 2000).

Most GEFs contain a Dbl homology (DH) domain, which interacts with the effector domain (or switch region) of the small GTPase. However, some (around 10) GEFs do not contain DH domains and therefore bind to the GTPase on other locations (Schmidt and Hall, 2002). Evidence demonstrates that GEFs tightly bind to the effector domain, but interact with other domains in the protein structure (Schmidt and Hall, 2002). The insert region is one such region that binds regions of certain GEFs and determines binding affinity (Schmidt and Hall, 2002). Because the insert region of Rac1 varies from that of Rac3, it is possible (and likely) that there are GEFs that preferentially bind one isoform over the other. This is an area of study that needs to be addressed in the future.

To elucidate differential GEF binding, we plan to use the stable cell lines created earlier that express the dominant negative Rac1 or Rac3 isoforms. Because the dominant negative isoforms are always in the inactive state, or constitutively bound to GDP, we hypothesize that GEFs will preferentially bind proteins in this state and try to activate them. However, this interaction will not be successful, creating an extension of the time period to which GEFs are bound to the GTPases. Subsequent to expression of the Rac mutant isoforms, an anti-Rac1 or anti-Rac3 immunoprecipitation will be performed on the cell lysates. Proteins from these immunoprecipitations will be elecrophoresed on 2-dimensional gels. Spots that differ between Rac1 immunoprecipitations and Rac3 immunoprecipitations will be excised and subjected to matrix-assisted laser desorption/ionization in a time-of-flight instrument (MALDI-TOF spectrometry). This

procedure will determine if there are any differences in GEF binding, and which GEFs preferentially bind the different isoforms of Rac.

Summary

In summary, the research presented here encompasses several aspects of cancer, cell, and molecular biology. Firstly, this research is important to the fields of cell signaling and cell biology. Signaling aspects of the Rho GTPases, as well as PTEN/PIP3/FAK interactions are addressed and explored. Additionally, the cellular processes of adhesion, cell migration, and invasion are investigated and their relation to signaling and implications for cancer metastasis are considered. Finally, this research makes a significant contribution to the field of cancer biology. Because of the necessity for more efficient anti-cancer therapies, it is essential to focus on basic science to identify molecular resources that can be tapped for better treatments.

BIBLIOGRAPHY

Abercrombie, M., Heaysman, J.E., and Pegrum, S.M. (1970). The locomotion of fibroblasts in culture. 3. Movements of particles on the dorsal surface of the leading lamella. Exp Cell Res *62*, 389-398.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. (2002) The Cytoskeleton and Cell Behavior. In: *Molecular Biology of The Cell, fourth edition.* Garland Science, NY, pp. 969-976.

Ali, I.U., Schriml, L.M., and Dean, M. (1999). Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity. J Natl Cancer Inst *91*, 1922-1932.

American Cancer Society. Cancer Facts and Figures 2005. *http://www.cancer.org /docroot/PRO/content/PRO_1_1_Cancer_Statistics_2005_Presentation.asp* [accessed 2005 March 29]

Anderson, E. (2004). Cellular homeostasis and the breast. Maturitas *48 Suppl 1*, S13-S17

Aspenstrom, P., Fransson, A., and Saras, J. (2004). Rho GTPases have diverse effects on the organization of the actin filament system. Biochem J *377*, 327-337.

Benard, V., Bohl, B.P., and Bokoch, G.M. (1999). Characterization of Rac and Cdc42 activation in chemoattractant- stimulated human neutrophils using a novel assay for active GTPases. J Biol Chem *274*, 13198-13204.

Beningo, K.A., Dembo, M., Kaverina, I., Small, J.V., and Wang, Y.L. (2001). Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. J Cell Biol J *153*, 881-888.

Benitah, S.A., Valeron, P.F., and Lacal, J.C. (2003). ROCK and nuclear factor-kappaB-dependent activation of cyclooxygenase-2 by Rho GTPases: effects on tumor growth and therapeutic consequences. Mol Biol Cell *14*, 3041-3054.

Benitah, S.A., Valeron, P.F., Van Aelst, L., Marshall, C.J., and Lacal, J.C. (2004). Rho GTPases in human cancer: an unresolved link to upstream and downstream transcriptional regulation. Biochim Biophys Acta *1705*, 121-132.

Besson, A., Robbins, S.M., and Yong, V.W. (1999). PTEN/MMAC1/TEP1 in signal transduction and tumorigenesis. Eur J Biochem *263*, 605-611.

Bibby, MC. (2004). Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. Eur J Cancer *40(6),* 852-857.

Bishop, A.L. and Hall, A. (2000). Rho GTPases and their effector proteins. Biochem J *348 Pt 2*, 241-255.

Bokoch, G.M. (2000). Regulation of cell function by Rho family GTPases. Immunol Res *21*, 139-148.

Bolis, A., Corbetta, S., Cioce, A., and de Curtis, I. (2003). Differential distribution of Rac1 and Rac3 GTPases in the developing mouse brain: implications for a role of Rac3 in Purkinje cell differentiation. Eur J Neurosci *18*, 2417-2424.

Bonneau, D. and Longy, M. (2000). Mutations of the human PTEN gene. Hum Mutat *16*, 109-122.

Bowcock, AM. (1999). Preface. In: *Breast Cancer: Molecular Genetics, Pathogenesis, and Therapeutics*. (Bowcock, AM., ed.). Humana Press, Totawa, NJ, pp.*v-vi.*

Bose, S., Crane, A., Hibshoosh, H., Mansukhani, M., Sandweis, L., and Parsons, R. (2002). Reduced expression of PTEN correlates with breast cancer progression. Hum Pathol *33*, 405-409.

Bourguignon, L.Y., Zhu, H., Shao, L., and Chen, Y.W. (2000). Ankyrin-Tiam1 interaction promotes Rac1 signaling and metastatic breast tumor cell invasion and migration. J Cell Biol *150*, 177-191.

Bouzahzah, B., Albanese, C., Ahmed, F., Pixley, F., Lisanti, M.P., Segall, J.D., Condeelis, J., Joyce, D., Minden, A., Der, C.J., Chan, A., Symons, M., and Pestell, R.G. (2001). Rho family GTPases regulate mammary epithelium cell growth and metastasis through distinguishable pathways. Mol Med *7*, 816-830.

Brakebusch, C., Bouvard, D., Stanchi, F., Sakai, T., and Fassler, R. (2002). Integrins in invasive growth. J Clin Invest *109*, 999-1006.

Brakebusch, C., Hirsch, E., Potocnik, A., and Fassler, R. (1997). Genetic analysis of beta1 integrin function: confirmed, new and revised roles for a crucial family of cell adhesion molecules. J Cell Sci *110(23)*, 2895-2904.

Brockbank, E.C., Bridges, J., Marshall, C.J., and Sahai, E. (2005). Integrin beta1 is required for the invasive behaviour but not proliferation of squamous cell carcinoma cells in vivo. Br J Cancer *92*, 102-112.

Brunton, V.G., MacPherson, I.R., and Frame, M.C. (2004). Cell adhesion receptors, tyrosine kinases and actin modulators: a complex three-way circuitry. Biochim Biophys Acta *1692*, 121-144.

Burridge, K. and Wennerberg, K. (2004). Rho and Rac take center stage. Cell *116*, 167-179.

Cantley, L.C. and Neel, B.G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci U S A *96*, 4240-4245.

Caponigro, F. (2002). Farnesyl transferase inhibitors: a major breakthrough in anticancer therapy? Anticancer Drugs *13*, 891-897.

Carragher, N.O. and Frame, M.C. (2004). Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion. Trends Cell Biol *14*, 241-249.

Cavallaro, U. and Christofori, G. (2004). Multitasking in tumor progression: signaling functions of cell adhesion molecules. Ann N Y Acad Sci *1014*, 58-66.

Chambers, A.F., Groom, A.C., and MacDonald, I.C. (2002). Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer *2*, 563-572.

Chambers, A.F., Naumov, G.N., Vantyghem, S.A., and Tuck, A.B. (2000). Molecular biology of breast cancer metastasis: Clinical implications of experimental studies on metastatic inefficiency. Breast Cancer Res *2*, 400-407.

Cho, S.Y. and Klemke, R.L. (2000). Extracellular-regulated kinase activation and CAS/Crk coupling regulate cell migration and suppress apoptosis during invasion of the extracellular matrix. J Cell Biol *149*, 223-236.

Chou, M.M. and Blenis, J. (1996). The 70 kDa S6 kinase complexes with and is activated by the Rho family G proteins Cdc42 and Rac1. Cell *85*, 573-583.

Chung, C.Y. and Firtel, R.A. (2002). Signaling pathways at the leading edge of chemotaxing cells. J Muscle Res Cell Motil *23*, 773-779.

Chung, M.J., Jung, S.H., Lee, B.J., Kang, M.J., and Lee, D.G. (2004). Inactivation of the PTEN gene protein product is associated with the invasiveness and metastasis, but not angiogenesis, of breast cancer. Pathol Int *54*, 10-15.

Clark, E.A., Golub, T.R., Lander, E.S., and Hynes, R.O. (2000). Genomic analysis of metastasis reveals an essential role for RhoC. Nature *406*, 532-535.

Clark, E.A., King, W.G., Brugge, J.S., Symons, M., and Hynes, R.O. (1998). Integrin-mediated signals regulated by members of the rho family of GTPases. J Cell Biol *142*, 573-586.

Condeelis, J.S., Wyckoff, J.B., Bailly, M., Pestell, R., Lawrence, D., Backer, J., and Segall, J.E. (2001). Lamellipodia in invasion. Semin Cancer Biol *11*, 119-128.

Cooper, GM., and Hausman, RE. (2004). Cancer. In: *The Cell: A Molecular Approach, edition three.* ASM Press, Washington, D.C., pp.631-673.

Cotteret, S. and Chernoff, J. (2002). The evolutionary history of effectors downstream of Cdc42 and Rac. Genome Biol *3(2)*, REVIEWS0002

Cox, E.A. and Huttenlocher, A. (1998). Regulation of integrin-mediated adhesion during cell migration. Microsc Res Tech *43*, 412-419.

Cukierman, E., Pankov, R., Stevens, D.R., and Yamada, K.M. (2001). Taking cell-matrix adhesions to the third dimension. Science *294*, 1708-1712.

Dahia, P.L. (2000). PTEN, a unique tumor suppressor gene. Endocr Relat Cancer *7*, 115-129.

Dangerfield, J.P., Wang, S., and Nourshargh, S. (2005). Blockade of alpha6 integrin inhibits IL-1beta- but not TNF-alpha-induced neutrophil transmigration in vivo. J Leukoc Biol *77*, 159-165.

Das, S., Dixon, J.E., and Cho, W. (2003). Membrane-binding and activation mechanism of PTEN. Proc Natl Acad Sci U S A *100*, 7491-7496.

Dinauer, M.C. (2003). Regulation of neutrophil function by Rac GTPases. Curr Opin Hematol *10*, 8-15.

Eden, S., Rohatgi, R., Podtelejnikov, A.V., Mann, M., and Kirschner, M.W. (2002). Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. Nature *418*, 790-793.

Egeblad, M. and Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer *2*, 161-174.

Eng, C. (2000). Will the real Cowden syndrome please stand up: revised diagnostic criteria. J Med Genet *37*, 828-830.

Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. Nature *420*, 629-635.

Evers, E.E., Zondag, G.C., Malliri, A., Price, L.S., ten Klooster, J.P., van der Kammen, R.A., and Collard, J.G. (2000). Rho family proteins in cell adhesion and cell migration. Eur J Cancer *36*, 1269-1274.

Folgueras, A.R., Pendas, A.M., Sanchez, L.M., and Lopez-Otin, C. (2004). Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. Int J Dev Biol *48*, 411-424.

Freeman, J.L., Abo, A., and Lambeth, J.D. (1996). Rac "insert region" is a novel effector region that is implicated in the activation of NADPH oxidase, but not PAK65. J Biol Chem *271*, 19794-19801.

Friedrichs, K., Ruiz, P., Franke, F., Gille, I., Terpe, H.J., and Imhof, B.A. (1995). High expression level of alpha 6 integrin in human breast carcinoma is correlated with reduced survival. Cancer Res *55*, 901-906.

Fritz, G., Brachetti, C., Bahlmann, F., Schmidt, M., and Kaina, B. (2002). Rho GTPases in human breast tumours: expression and mutation analyses and correlation with clinical parameters. Br J Cancer *87*, 635-644.

Fritz, G., Just, I., and Kaina, B. (1999). Rho GTPases are over-expressed in human tumors. Int J Cancer *81*, 682-687.

Funamoto, S., Meili, R., Lee, S., Parry, L., and Firtel, R.A. (2002). Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. Cell *109*, 611-623.

Gautam, A., Li, Z.R., and Bepler, G. (2003). RRM1-induced metastasis suppression through PTEN-regulated pathways. Oncogene *22*, 2135-2142.

Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K.M. (2001). Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. Nat Rev Mol Cell Biol *2*, 793-805.

Gildea, J.J., Seraj, M.J., Oxford, G., Harding, M.A., Hampton, G.M., Moskaluk, C.A., Frierson, H.F., Conaway, M.R., and Theodorescu, D. (2002). RhoGDI2 is an invasion and metastasis suppressor gene in human cancer. Cancer Res *62*, 6418-6423.

Gimond, C., van Der, F., van Delft, S., Brakebusch, C., Kuikman, I., Collard, J.G., Fassler, R., and Sonnenberg, A. (1999). Induction of cell scattering by expression of beta1 integrins in beta1- deficient epithelial cells requires activation of members of the rho family of GTPases and downregulation of cadherin and catenin function. J Cell Biol *147*, 1325-1340.

Glacy, S.D. (1983). Subcellular distribution of rhodamine-actin microinjected into living fibroblastic cells. J Cell Biol *97*, 1207-1213.

Goberdhan, D.C. and Wilson, C. (2003). PTEN: tumour suppressor, multifunctional growth regulator and more. Hum Mol Genet *12 Spec No 2*, R239-R248

Gu, J., Tamura, M., Pankov, R., Danen, E.H., Takino, T., Matsumoto, K., and Yamada, K.M. (1999). Shc and FAK differentially regulate cell motility and directionality modulated by PTEN. J Cell Biol *146*, 389-403.

Gu, J., Tamura, M., and Yamada, K.M. (1998). Tumor suppressor PTEN inhibits integrin- and growth factor-mediated mitogen-activated protein (MAP) kinase signaling pathways. J Cell Biol *143*, 1375-1383.

Haataja, L., Groffen, J., and Heisterkamp, N. (1997). Characterization of RAC3, a novel member of the Rho family. J Biol Chem *272*, 20384-20388.

Haataja, L., Kaartinen, V., Groffen, J., and Heisterkamp, N. (2002). The small GTPase Rac3 interacts with the integrin-binding protein CIB and promotes integrin alpha(IIb)beta(3)-mediated adhesion and spreading. J Biol Chem *277*, 8321-8328.

Haeusler, L.C., Blumenstein, L., Stege, P., Dvorsky, R., and Ahmadian, M.R. (2003). Comparative functional analysis of the Rac GTPases. FEBS Lett *555*, 556-560.

Hall, A. and Nobes, C.D. (2000). Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. Philos Trans R Soc Lond B Biol Sci *355*, 965-970.

Higgs, H.N. and Pollard, T.D. (2001). Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. Annu Rev Biochem *70*, 649-676.

Hintermann, E. and Quaranta, V. (2004). Epithelial cell motility on laminin-5: regulation by matrix assembly, proteolysis, integrins and erbB receptors. Matrix Biol *23*, 75-85.

Hirsch, E., Barberis, L., Brancaccio, M., Azzolino, O., Xu, D., Kyriakis, J.M., Silengo, L., Giancotti, F.G., Tarone, G., Fassler, R., and Altruda, F. (2002). Defective Rac-

mediated proliferation and survival after targeted mutation of the beta1 integrin cytodomain. J Cell Biol *157*, 481-492.

Hynes, R.O. (2002). Integrins: bidirectional, allosteric signaling machines. Cell *110*, 673-687.

Iijima, M. and Devreotes, P. (2002). Tumor suppressor PTEN mediates sensing of chemoattractant gradients. Cell *109*, 599-610.

Iijima, M., Huang, Y.E., and Devreotes, P. (2002). Temporal and spatial regulation of chemotaxis. Dev Cell *3*, 469-478.

Itoh, K., Yoshioka, K., Akedo, H., Uehata, M., Ishizaki, T., and Narumiya, S. (1999). An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. Nat Med *5*, 221-225.

Jaffe, A.B. and Hall, A. (2002). Rho GTPases in transformation and metastasis. Adv Cancer Res *84*, 57-80.

Jakobisiak, M. and Golab, J. (2003). Potential antitumor effects of statins. Int J Oncol *23*, 1055-1069.

Jiang, F.X., Georges-Labouesse, E., and Harrison, L.C. (2001). Regulation of laminin 1-induced pancreatic beta-cell differentiation by alpha6 integrin and alpha-dystroglycan. Mol Med *7*, 107-114.

Jiang, W.G., Watkins, G., Fodstad, O., Douglas-Jones, A., Mokbel, K., and Mansel, R.E. (2004). Differential expression of the CCN family members Cyr61, CTGF and Nov in human breast cancer. Endocr Relat Cancer *11*, 781-791.

Joyce, P.L. and Cox, A.D. (2003). Rac1 and Rac3 are targets for geranylgeranyltransferase I inhibitor-mediated inhibition of signaling, transformation, and membrane ruffling. Cancer Res *63* , 7959-7967.

Kamai, T., Tsujii, T., Arai, K., Takagi, K., Asami, H., Ito, Y., and Oshima, H. (2003). Significant association of Rho/ROCK pathway with invasion and metastasis of bladder cancer. Clin Cancer Res *9*, 2632-2641.

Kandel, E.S. and Hay, N. (1999). The regulation and activities of the multifunctional Serine/Threonine kinase Akt/PKB. Exp Cell Res *253*, 210-229.

Karp. G. (1999). Cancer. In: *Cell and Molecular Biology*. John Wiley and Sons, Inc., NY, pp.700-725.

Kassis, J., Lauffenburger, D.A., Turner, T., and Wells, A. (2001). Tumor invasion as dysregulated cell motility. Semin Cancer Biol *11*, 105-117.

Katoh, K., Kano, Y., Amano, M., Onishi, H., Kaibuchi, K., and Fujiwara, K. (2001). Rho-kinase--mediated contraction of isolated stress fibers. J Cell Biol *153*, 569-584.

Kaverina, I., Krylyshkina, O., and Small, J.V. (2002). Regulation of substrate adhesion dynamics during cell motility. Int J Biochem Cell Biol *34*, 746-761.

Keller, E.T. (2004). Metastasis suppressor genes: a role for raf kinase inhibitor protein (RKIP). Anticancer Drugs *15*, 663-669.

Khanna, C., Hunter, K. (2005). Modeling metastasis in vivo. Carcinogenesis *26(3),* 513-23.

Khanna, C., Prehn, J., Yeung, C., Caylor, J., Tsokos, M., and Helman, L. (2000). An orthotopic model of murine osteosarcoma with clonally related variants differing in pulmonary metastatic potential. Clin Exp Metastasis *18(3),* 261-71.

Kim, M.S., Park, M.J., Moon, E.J., Kim, S.J., Lee, C.H., Yoo, H., Shin, S.H., Song, E.S., and Lee, S.H. (2005). Hyaluronic acid induces osteopontin via the phosphatidylinositol 3-kinase/Akt pathway to enhance the motility of human glioma cells. Cancer Res *65*, 686-691.

Kleer, C.G., van Golen, K.L., Zhang, Y., Wu, Z.F., Rubin, M.A., and Merajver, S.D. (2002). Characterization of RhoC expression in benign and malignant breast disease: a potential new marker for small breast carcinomas with metastatic ability. Am J Pathol *160*, 579-584.

Kleiner, D.E. and Stetler-Stevenson, W.G. (1999). Matrix metalloproteinases and metastasis. Cancer Chemother Pharmacol *43 Suppl*, S42-S51

Klemke, R.L., Leng, J., Molander, R., Brooks, P.C., Vuori, K., and Cheresh, D.A. (1998). CAS/Crk coupling serves as a "molecular switch" for induction of cell migration. J Cell Biol *140*, 961-972.

Knaus, U.G., Heyworth, P.G., Kinsella, B.T., Curnutte, J.T., and Bokoch, G.M. (1992). Purification and characterization of Rac 2, A cytosolic GTP-binding protein that regulates human neutrophil NADPH oxidase. J Biol Chem *267*, 23575-23582.

Kondo, T., Sentani, K., Oue, N., Yoshida, K., Nakayama, H., and Yasui, W. (2004). Expression of RHOC is associated with metastasis of gastric carcinomas. Pathobiology *71*, 19-25.

Lara, P.N.J., Law, L.Y., Wright, J.J., Frankel, P., Twardowski, P., Lenz, H.J., Lau, D.H., Kawaguchi, T., Gumerlock, P.H., Doroshow, J.H., and Gandara, D.R. (2005). Intermittent dosing of the farnesyl transferase inhibitor tipifarnib (R115777) in advanced malignant solid tumors: a phase I California Cancer Consortium Trial. Anticancer Drugs *16*, 317-321.

Lee, J.O., Yang, H., Georgescu, M.M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfi, P., and Pavletich, N.P. (1999). Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. Cell *99*, 323-334.

Lee, J.S., Kim, H.S., Kim, Y.B., Lee, M.C., Park, C.S., and Min, K.W. (2004). Reduced PTEN expression is associated with poor outcome and angiogenesis in invasive

ductal carcinoma of the breast. Appl Immunohistochem Mol Morphol *12*, 205-210.

Lee, T.K., Man, K., Ho, J.W., Wang, X.H., Poon, R.T., Sun, K.W., Ng, K.T., Ng, I.O., Xu, R., and Fan, S.T. (2004). The significance of Rac signaling pathway in HCC cell motility: implication for new therapeutic target. Carcinogenesis *26(3):*681-687.

Leslie, N.R. and Downes, C.P. (2002). PTEN: The down side of PI 3-kinase signalling. Cell Signal *14*, 285-295.

Leslie, N.R. and Downes, C.P. (2004). PTEN function: how normal cells control it and tumour cells lose it. Biochem J *382*, 1-11.

Leung, K., Nagy, A., Gonzalez-Gomez, I., Groffen, J., Heisterkamp, N., and Kaartinen, V. (2003). Targeted expression of activated Rac3 in mammary epithelium leads to defective postlactational involution and benign mammary gland lesions. Cells Tissues Organs *175*, 72-83.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H., and Parsons, R. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science *275*, 1943-1947.

Liliental, J., Moon, S.Y., Lesche, R., Mamillapalli, R., Li, D., Zheng, Y., Sun, H., and Wu, H. (2000). Genetic deletion of the Pten tumor suppressor gene promotes cell motility by activation of Rac1 and Cdc42 GTPases. Curr Biol *10*, 401-404.

Lozano, E., Betson, M., and Braga, V.M. (2003). Tumor progression: Small GTPases and loss of cell-cell adhesion. Bioessays *25*, 452-463.

Maehama, T. and Dixon, J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem *273*, 13375-13378.

Maehama, T., Okahara, F., and Kanaho, Y. (2004). The tumour suppressor PTEN: involvement of a tumour suppressor candidate protein in PTEN turnover. Biochem Soc Trans *32*, 343-347.

Marcoux, N. and Vuori, K. (2003). EGF receptor mediates adhesion-dependent activation of the Rac GTPase: a role for phosphatidylinositol 3-kinase and Vav2. Oncogene *22*, 6100-6106.

Martin, G.S. (2003). Cell signaling and cancer. Cancer Cell *4*, 167-174.

Martin, K.H., Slack, J.K., Boerner, S.A., Martin, C.C., and Parsons, J.T. (2002). Integrin connections map: to infinity and beyond. Science1 *296*, 1652-1653.

Matsudaira, P. (1994). Actin crosslinking proteins at the leading edge. Semin Cell Biol *5*, 165-174.

Mercurio, A.M., Bachelder, R.E., Rabinovitz, I., O'Connor, K.L., Tani, T., and Shaw, L.M. (2001). The metastatic odyssey: the integrin connection. Surg Oncol Clin N Am *10*, 313-3ix.

Miao, H., Li, S., Hu, Y.L., Yuan, S., Zhao, Y., Chen, B.P., Puzon-McLaughlin, W., Tarui, T., Shyy, J.Y., Takada, Y., Usami, S., and Chien, S. (2002). Differential regulation of Rho GTPases by beta1 and beta3 integrins: the role of an extracellular domain of integrin in intracellular signaling. J Cell Sci *115*, 2199-2206.

Miki, H. and Takenawa, T. (2003). Regulation of actin dynamics by WASP family proteins. J Biochem (Tokyo) *134* , 309-313.

Mira, J.P., Benard, V., Groffen, J., Sanders, L.C., and Knaus, U.G. (2000). Endogenous, hyperactive Rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway. Proc Natl Acad Sci U S A *97*, 185-189.

Miranti, C.K. and Brugge, J.S. (2002). Sensing the environment: a historical perspective on integrin signal transduction. Nat Cell Biol *4*, E83-E90

Mitra, S.K., Hanson, D.A., and Schlaepfer, D.D. (2005). Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol *6*, 56-68.

Miyamoto, S., Teramoto, H., Gutkind, J.S., and Yamada, K.M. (1996). Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases

and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. J Cell Biol *135*, 1633-1642.

Moro, L., Venturino, M., Bozzo, C., Silengo, L., Altruda, F., Beguinot, L., Tarone, G., and Defilippi, P. (1998). Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. EMBO J *17*, 6622-6632.

Mukhopadhyay, R., Theriault, R.L., and Price, J.E. (1999). Increased levels of alpha6 integrins are associated with the metastatic phenotype of human breast cancer cells. Clin Exp Metastasis *17*, 325-332.

Myers, M.P., Stolarov, J.P., Eng, C., Li, J., Wang, S.I., Wigler, M.H., Parsons, R., and Tonks, N.K. (1997). P-TEN, the tumor suppressor from human chromosome 10q23, is a dual- specificity phosphatase. Proc Natl Acad Sci U S A *94*, 9052-9057.

Nheu, T.V., He, H., Hirokawa, Y., Tamaki, K., Florin, L., Schmitz, M.L., Suzuki-Takahashi, I., Jorissen, R.N., Burgess, A.W., Nishimura, S., Wood, J., and Maruta, H. (2002). The K252a derivatives, inhibitors for the PAK/MLK kinase family selectively block the growth of RAS transformants. Cancer J *8*, 328-336.

Nobes, C.D. and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell *81*, 53-62.

Nobes, C.D. and Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. J Cell Biol *144*, 1235-1244.

Nobes, C.D., Lauritzen, I., Mattei, M.G., Paris, S., Hall, A., and Chardin, P. (1998). A new member of the Rho family, Rnd1, promotes disassembly of actin filament structures and loss of cell adhesion. J Cell Biol *141*, 187-197.

O'Connor, K.L. and Mercurio, A.M. (2001). Protein kinase A regulates Rac and is required for the growth factor-stimulated migration of carcinoma cells. J Biol Chem *276*, 47895-47900.

O'Connor, K.L., Nguyen, B.K., and Mercurio, A.M. (2000). RhoA function in lamellae formation and migration is regulated by the alpha6beta4 integrin and cAMP metabolism. J Cell Biol *148*, 253-258.

Otsuki, Y., Tanaka, M., Yoshii, S., Kawazoe, N., Nakaya, K., and Sugimura, H. (2001). Tumor metastasis suppressor nm23H1 regulates Rac1 GTPase by interaction with Tiam1. Proc Natl Acad Sci U S A *98*, 4385-4390.

Overall, C.M. and Lopez-Otin, C. (2002). Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer *2*, 657-672.

Pandolfi, P.P. (2004). Breast cancer--loss of PTEN predicts resistance to treatment. N Engl J Med *351*, 2337-2338.

Parsons, R. and Simpson, L. (2003). PTEN and cancer. Methods Mol Biol *222*, 147-166.

Parsons, S.J. and Parsons, J.T. (2004). Src family kinases, key regulators of signal transduction. Oncogene *23*, 7906-7909.

Payrastre, B., Missy, K., Giuriato, S., Bodin, S., Plantavid, M., and Gratacap, M. (2001). Phosphoinositides: key players in cell signalling, in time and space. Cell Signal *13*, 377-387.

Perren, A., Weng, L.P., Boag, A.H., Ziebold, U., Thakore, K., Dahia, P.L., Komminoth, P., Lees, J.A., Mulligan, L.M., Mutter, G.L., and Eng, C. (1999). Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. Am J Pathol *155*, 1253-1260.

Petit, V. and Thiery, J.P. (2000). Focal adhesions: structure and dynamics. Biol Cell *92*, 477-494.

Pille, J.Y., Denoyelle, C., Varet, J., Bertrand, J.R., Soria, J., Opolon, P., Lu, H., Pritchard, L.L., Vannier, J.P., Malvy, C., Soria, C., and Li, H. (2005). Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo. Mol Ther *11*, 267-274.

Playford, M.P. and Schaller, M.D. (2004). The interplay between Src and integrins in normal and tumor biology. Oncogene *23*, 7928-7946.

Pollard, T.D. and Beltzner, C.C. (2002). Structure and function of the Arp2/3 complex. Curr Opin Struct Biol *12*, 768-774.

Prendergast, G.C. (2001). Actin' up: RhoB in cancer and apoptosis. Nat Rev Cancer *1*, 162-168.

Price, L.S. and Collard, J.G. (2001). Regulation of the cytoskeleton by Rho-family GTPases: implications for tumour cell invasion. Semin Cancer Biol *11*, 167-173.

Pu, P., Kang, C., Li, J., and Jiang, H. (2004). Antisense and dominant-negative AKT2 cDNA inhibits glioma cell invasion. Tumour Biol *25*, 172-178.

Quaranta, V. and Giannelli, G. (2003). Cancer invasion: watch your neighbourhood! Tumori *89*, 343-348.

Rameh, L.E. and Cantley, L.C. (1999). The role of phosphoinositide 3-kinase lipid products in cell function. J Biol Chem *274*, 8347-8350.

Ren, X.D. and Schwartz, M.A. (2000). Determination of GTP loading on Rho. Methods Enzymol *325*, 264-272.

Ridley, A.J. (1997). The GTP-binding protein Rho. Int J Biochem Cell Biol *29*, 1225-1229.

Ridley, A.J. (2001). Rho GTPases and cell migration. J Cell Sci *114*, 2713-2722.

Ridley, A.J. (2004). Rho proteins and cancer. Breast Cancer Res Treat *84*, 13-19.

Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T., and Horwitz, A.R. (2003). Cell migration: integrating signals from front to back. Science *302*, 1704-1709.

Riento, K. and Ridley, A.J. (2003). Rocks: multifunctional kinases in cell behaviour. Nat Rev Mol Cell Biol *4*, 446-456.

Rottner, K., Hall, A., and Small, J.V. (1999). Interplay between Rac and Rho in the control of substrate contact dynamics. Curr Biol *9*, 640-648.

Rubinfeld, H. and Seger, R. (2004). The ERK cascade as a prototype of MAPK signaling pathways. Methods Mol Biol *250*, 1-28.

Russo, J., Hu, Y.F., Silva, I.D., and Russo, I.H. (2001). Cancer risk related to mammary gland structure and development. Microsc Res Tech *52*, 204-223.

Sahai, E. and Marshall, C.J. (2002). RHO-GTPases and cancer. Nat Rev Cancer *2*, 133-142.

Sahai, E. and Marshall, C.J. (2002). ROCK and Dia have opposing effects on adherens junctions downstream of Rho. Nat Cell Biol *4*, 408-415.

Sakai, T., Peyruchaud, O., Fassler, R., and Mosher, D.F. (1998). Restoration of beta1A integrins is required for lysophosphatidic acid-induced migration of beta1-null mouse fibroblastic cells. J Biol Chem *273*, 19378-19382.

Sakai, T., Zhang, Q., Fassler, R., and Mosher, D.F. (1998). Modulation of beta1A integrin functions by tyrosine residues in the beta1 cytoplasmic domain. J Cell Biol *141*, 527-538.

Schaller, M.D. (2001). Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim Biophys Acta *1540*, 1-21.

Schlaepfer, D.D. and Mitra, S.K. (2004). Multiple connections link FAK to cell motility and invasion. Curr Opin Genet Dev *14*, 92-101.

Schlaepfer, D.D., Mitra, S.K., and Ilic, D. (2004). Control of motile and invasive cell phenotypes by focal adhesion kinase. Biochim Biophys Acta *1692*, 77-102.

Schmidt, A. and Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev *16*, 1587-1609.

Schmitz, A.A., Govek, E.E., Bottner, B., and Van Aelst, L. (2000). Rho GTPases: signaling, migration, and invasion. Exp Cell Res *261*, 1-12.

Schoenwaelder, S.M. and Burridge, K. (1999). Bidirectional signaling between the cytoskeleton and integrins. Curr Opin Cell Biol *11*, 274-286.

Shikada, Y., Yoshino, I., Okamoto, T., Fukuyama, S., Kameyama, T., and Maehara, Y. (2003). Higher expression of RhoC is related to invasiveness in non-small cell lung carcinoma. Clin Cancer Res *9*, 5282-5286.

Shimizu, H., Koyama, N., Asada, M., and Yoshimatsu, K. (2002). Aberrant expression of integrin and erbB subunits in breast cancer cell lines. Int J Oncol *21*, 1073-1079.

Shoman, N., Klassen, S., McFadden, A., Bickis, M.G., Torlakovic, E., and Chibbar, R. (2005). Reduced PTEN expression predicts relapse in patients with breast carcinoma treated by tamoxifen. Mod Pathol *18*, 250-259.

Simpson, K.J., Dugan, A.S., and Mercurio, A.M. (2004). Functional analysis of the contribution of RhoA and RhoC GTPases to invasive breast carcinoma. Cancer Res *64*, 8694-8701.

Sliva, D., Mason, R., Xiao, H., and English, D. (2000). Enhancement of the migration of metastatic human breast cancer cells by phosphatidic acid. Biochem Biophys Res Commun *268*, 471-479.

Smith, L.G. and Li, R. (2004). Actin polymerization: riding the wave. Curr Biol *14*, R109-R111

Srinivasan, S., Wang, F., Glavas, S., Ott, A., Hofmann, F., Aktories, K., Kalman, D., and Bourne, H.R. (2003). Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. J Cell Biol *160*, 375-385.

Stam, J.C., Michiels, F., van der Kammen, R.A., Moolenaar, W.H., and Collard, J.G. (1998). Invasion of T-lymphoma cells: cooperation between Rho family GTPases and lysophospholipid receptor signaling. EMBO J *17*, 4066-4074.

Stambolic, V., Mak, T.W., and Woodgett, J.R. (1999). Modulation of cellular apoptotic potential: contributions to oncogenesis. Oncogene *18*, 6094-6103.

Steeg, P.S. (2003). Metastasis suppressors alter the signal transduction of cancer cells. Nat Rev Cancer *3*, 55-63.

Steeg, P.S., Ouatas, T., Halverson, D., Palmieri, D., and Salerno, M. (2003). Metastasis suppressor genes: basic biology and potential clinical use. Clin Breast Cancer *4*, 51-62.

Steeg, P.S., Ouatas, T., Halverson, D., Palmieri, D., and Salerno, M. (2003). Metastasis suppressor genes: basic biology and potential clinical use. Clin Breast Cancer *4*, 51-62.

Stiles, B., Groszer, M., Wang, S., Jiao, J., and Wu, H. (2004). PTENless means more. Dev Biol *273*, 175-184.

Sturge, J., Hamelin, J., and Jones, G.E. (2002). N-WASP activation by a beta1-integrin-dependent mechanism supports PI3K-independent chemotaxis stimulated by urokinase-type plasminogen activator. J Cell Sci *115*, 699-711.

Sulis, M.L. and Parsons, R. (2003). PTEN: from pathology to biology. Trends Cell Biol *13*, 478-483.

Tagliabue, E., Ghirelli, C., Squicciarini, P., Aiello, P., Colnaghi, M.I., and Menard, S. (1998). Prognostic value of alpha 6 beta 4 integrin expression in breast

carcinomas is affected by laminin production from tumor cells. Clin Cancer Res *4*, 407-410.

Tamura, M., Gu, J., Danen, E.H., Takino, T., Miyamoto, S., and Yamada, K.M. (1999). PTEN interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway. J Biol Chem *274*, 20693-20703.

Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R., and Yamada, K.M. (1998). Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science *280*, 1614-1617.

Tamura, M., Gu, J., Tran, H., and Yamada, K.M. (1999). PTEN gene and integrin signaling in cancer. J Natl Cancer Inst *91*, 1820-1828.

Toker, A. (2000). Protein kinases as mediators of phosphoinositide 3-kinase signaling. Mol Pharmacol *57*, 652-658.

Uhlenbrock, K., Eberth, A., Herbrand, U., Daryab, N., Stege, P., Meier, F., Friedl, P., Collard, J.G., and Ahmadian, M.R. (2004). The RacGEF Tiam1 inhibits migration and invasion of metastatic melanoma via a novel adhesive mechanism. J Cell Sci *117*, 4863-4871.

Urtreger, A.J., Grossoni, V.C., Falbo, K.B., Kazanietz, M.G., and Bal de Kier Joffe ED. (2005). Atypical protein kinase C-zeta modulates clonogenicity, motility, and

secretion of proteolytic enzymes in murine mammary cells. Mol Carcinog *42*, 29-39.

Vadlamudi, R.K. and Kumar, R. (2003). P21-activated kinases in human cancer. Cancer Metastasis Rev *22*, 385-393.

van Golen, K.L., Davies, S., Wu, Z.F., Wang, Y., Bucana, C.D., Root, H., Chandrasekharappa, S., Strawderman, M., Ethier, S.P., and Merajver, S.D. (1999). A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype. Clin Cancer Res *5*, 2511-2519.

van Golen, K.L., Wu, Z.F., Qiao, X.T., Bao, L.W., and Merajver, S.D. (2000). RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. Cancer Res *60*, 5832-5838.

van Leeuwen, F.N., van der Kammen, R.A., Habets, G.G., and Collard, J.G. (1995). Oncogenic activity of Tiam1 and Rac1 in NIH3T3 cells. Oncogene *11*, 2215-2221.

Vasko, V., Saji, M., Hardy, E., Kruhlak, M., Larin, A., Savchenko, V., Miyakawa, M., Isozaki, O., Murakami, H., Tsushima, T., Burman, K.D., De Micco, C., and Ringel, M.D. (2004). Akt activation and localisation correlate with tumour invasion and oncogene expression in thyroid cancer. J Med Genet *41*, 161-170.

Vazquez, F. and Sellers, W.R. (2000). The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3-kinase signaling. Biochim Biophys Acta *1470*, M21-M35

Vial, E., Sahai, E., and Marshall, C.J. (2003). ERK-MAPK signaling coordinately regulates activity of Rac1 and RhoA for tumor cell motility. Cancer Cell *4*, 67-79.

Waite, K.A. and Eng, C. (2002). Protean PTEN: form and function. Am J Hum Genet *70*, 829-844.

Wallar, B.J. and Alberts, A.S. (2003). The formins: active scaffolds that remodel the cytoskeleton. Trends Cell Biol *13*, 435-446.

Watts, A.M. and Kennedy, R.C. (1998). Quantitation of tumor foci in an experimental murine tumor model using computer-assisted video imaging. Anal Biochem *256*, 217-219.

Webb, D.J., Donais, K., Whitmore, L.A., Thomas, S.M., Turner, C.E., Parsons, J.T., and Horwitz, A.F. (2004). FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol *6*, 154-161.

Wedlich-Soldner, R., Altschuler, S., Wu, L., and Li, R. (2003). Spontaneous cell polarization through actomyosin-based delivery of the Cdc42 GTPase. Science *299*, 1231-1235.

Wennerberg, K. and Der, C.J. (2004). Rho-family GTPases: it's not only Rac and Rho (and I like it). J Cell Sci *117*, 1301-1312.

Westwick, J.K., Lambert, Q.T., Clark, G.J., Symons, M., Van Aelst, L., Pestell, R.G., and Der, C.J. (1997). Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. Mol Cell Biol *17*, 1324-1335.

Wewer, U.M., Shaw, L.M., Albrechtsen, R., and Mercurio, A.M. (1997). The integrin alpha 6 beta 1 promotes the survival of metastatic human breast carcinoma cells in mice. Am J Pathol *151*, 1191-1198.

Wheeler, A.P. and Ridley, A.J. (2004). Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. Exp Cell Res *301*, 43-49.

Wood, W. and Martin, P. (2002). Structures in focus--filopodia. Int J Biochem Cell Biol *34*, 726-730.

Wozniak, M.A., Modzelewska, K., Kwong, L., and Keely, P.J. (2004). Focal adhesion regulation of cell behavior. Biochim Biophys Acta *1692*, 103-119.

Yamada, K.M. and Araki, M. (2001). Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. J Cell Sci *114*, 2375-2382.

Yamada, K.M., Pankov, R., and Cukierman, E. (2003). Dimensions and dynamics in integrin function. Braz J Med Biol Res *36*, 959-966.

Zamir, E. and Geiger, B. (2001). Molecular complexity and dynamics of cell-matrix adhesions. J Cell Sci *114*, 3583-3590.

Zhan, M., Zhao, H., and Han, Z.C. (2004). Signalling mechanisms of anoikis. Histol Histopathol *19*, 973-983.

Zhang, L., Yu, Q., He, J., and Zha, X. (2004). Study of the PTEN gene expression and FAK phosphorylation in human hepatocarcinoma tissues and cell lines. Mol Cell Biochem *262*, 25-33.

Zhang, L.N., Yu, Q., Wang, L.Y., Jin, J.W., and Zha, X.L. (2003). [The effects of PTEN gene on migration and FAK phosphorylation of SMMC-7721 human hepatocarcinoma cell line]. Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai) *35*, 161-166.

Zhuge, Y. and Xu, J. (2001). Rac1 mediates type i collagen-dependent mmp-2 activation. role in cell invasion across collagen barrier. J Biol Chem *276*, 16248-16256.

VITA

Paige Jennette Baugher was born in Huntsville, Alabama, on September 1, 1976, the daughter of Charles and Patty R. Baugher. After earning an Advanced Diploma from Virgil I. Grissom High School, she entered Vanderbilt University in Nashville, Tennessee. She received a Bachelor of Music Degree in Clarinet Performance with a double major in Molecular Biology from Vanderbilt University in December of 1998. During the following year, she worked as a research assistant in the Neuroscience Department of Vanderbilt University. In August of 1999, she entered graduate school at the University of Texas at Austin as a pre-emptive fellow in the Botany Department. In August of 2000, she transferred into the Molecular Biology Department at the University of Texas at Austin.

Permanent Address: PO Box 4212, Huntsville, AL, 35815

This dissertation was typed by the author.